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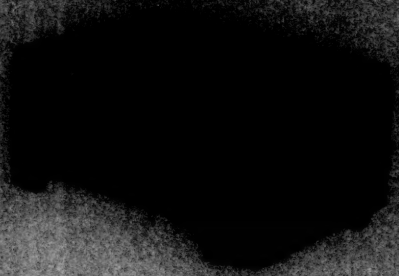

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THE CALCIUM CONTENT OF HUMAN BLOOD.

By HENRY LYMAN.

(From the Research Laboratory of the Huntington Memorial Hospital, and the Biochemical Laboratory of the Harvard Medical School, Boston.)

(Received for publication, March 29, 1917.)

The accompanying table gives the result of the analyses for calcium of fifty-four samples of human blood, determinations of the total non-coagulable nitrogen also being done on the pathological cases. It is hoped that these figures may help to establish a base line for further study of the metabolism of this metal. The chief point brought out seems to be that the calcium varies but little in a great majority of subjects. Even in cases of advanced uremia, where the total nitrogen was extremely high, the calcium was not increased. On the other hand, the hemophiliac and the case of purpura hemorrhagica were not lower than what we may regard as normal.

The average for the males, with the exception of No. 36, was 6.1 mg. of calcium per 100 cc. of blood; while that for females was 7.1, a difference sufficiently great to stimulate further investigation.

In No. 36 there seems to be no reason for the extremely low figure shown. Duplicate determinations were of course performed and there is no doubt that this represents the correct calcium content. Unfortunately, as the patient soon left the hospital, a second specimen could not be obtained. No. 8, on the other hand, was taken from a healthy young woman whose history gives us no clue to the explanation of the high result—9.6 mg. per 100 cc.

Determination of calcium was carried out according to the new method of the author,[1] while the total nitrogens were done by the direct Nesslerization process of Folin and Denis.[2] As it was more convenient to perform both analyses in the same filtrate, the following technique was employed:

[1] Lyman, H., *J. Biol. Chem.*, 1917, xxix, 169.

[2] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 473.

Blood was drawn into a 20 cc. paraffined pipette and run into a flask containing 55 cc. of distilled water. After agitating to lake the blood, 5 cc. of 80 per cent trichloroacetic acid were added, the whole well shaken, and allowed to stand for an hour or longer, as convenient. After filtering through calcium-free filter paper, 10 cc. of the filtrate were used for the calcium determinations and 5 cc. for the total nitrogens. The coagulation by trichloroacetic acid was first carefully checked by the phosphoric acid method of Folin and Denis and found to give identical results.

Not only does this procedure lessen the number of flasks and solutions to be carried about, but it also shortens the time of the nitrogen determinations, as there are only 5 cc. of fluid to boil off instead of 10 cc. Moreover, no pebble or bead need be used in the digestions as there is no bumping.

The specimens, with the exception of the normal cases, were obtained from the Massachusetts General Hospital, the Peter Bent Brigham Hospital, and the Huntington Memorial Hospital.

TABLE I.

Calcium and Total Non-Coagulable Nitrogen per 100 Cc. of Human Blood.

Case.	Sex.	Diagnosis.	Ca	N
			mg.	*mg.*
1	M.	Normal.	6.1	
2	"	"	6.2	
3	"	Chronic infectious arthritis.	5.9	
4	"	Normal.	6.1	
5	"	"	6.1	
6	F.	"	7.2	
7	"	Acromegaly.	7.3	
8	"	Normal.	9.6	
9	"	"	7.7	
10	M.	"	6.1	
11	"	"	6.4	
12	"	"	6.7	
13	"	"	5.2	
14	"	Carcinoma of jaw.	6.4	
15	"	" " larynx.	6.0	
16	"	" " eyelid.	6.4	
17	"	Hemophiliac.	6.0	39.4
18	"	Purpura hemorrhagica.	6.4	37.0
19	"	Myelogenous leukemia.	6.4	61.8
20	"	Cardiac.	6.1	52.0
21	"	Nephritis.	6.0	56.6
22	"	Hyperthyroid.	6.9	41.0

TABLE I—*Concluded.*

Case.	Sex.	Diagnosis.	Ca	N
			mg.	*mg.*
23	M.	Diabetic (16 yrs.).	4.8	32.6
24	"	Syphilis.	6.5	35.6
25	"	Amebic dysentery.	4.2	32.0
26	"	Tumor of orbit, malignant (?).	5.3	41.0
27	"	Myelogenous leukemia.	6.2	47.4
28	"	Gout.	6.0	44.8
29	"	Syphilis.	5.5	36.2
30	"	Gout and uremia.	5.7	76.8
31	"	Pleurisy with effusion.	5.5	44.4
32	"	Nephritis.	6.6	49.2
33	"	Tuberculosis.	6.1	36.4
34	"	Cardiac, in extremis.	6.8	98.8
35	"	Cardiorenal with asthma.	6.4	50.0
36	"	Gout (?), congenital heart, hemoglobin 160 per cent.	2.8	37.8
37	"	Hyperthyroid.	6.4	32.0
38	F.	Chronic nephritis, uremia, clonic twitching.	5.2	134.4
39	"	Carcinoma (?) of stomach.	7.3	35.8
40	"	Diarrhea.	6.4	30.2
41	"	Multiple neuritis.	6.1	44.8
42	"	Hyperthyroid.	6.4	31.0
43	"	Diabetes.	6.7	29.2
44	"	Acute nephritis.	7.6	39.2
45	M.	Myelogenous leukemia.	6.3	45.6
46	"	Carcinoma of pharynx.	6.6	35.2
47	"	" " eyelid.	6.7	38.2
48	"	" " tongue.	6.9	31.0
49	"	Sarcoma of prostate.	6.5	30.8
50	F.	Carcinoma of rectum.	7.8	32.4
51	"	" " cervix.	6.8	39.2
52	"	" " "	7.4	25.2
53	M.	Sarcoma of glands of neck.	6.5	34.0
54	"	Normal.	5.5	

THE MECHANISM OF CYTOLYSIS IN ECHINODERM EGGS.

SECOND PAPER.

BY A. R. MOORE.

(*From the Physiological Laboratory of Rutgers College, New Brunswick, N. J., and the Marine Biological Laboratory, Woods Hole, Mass.*)

(Received for publication, March 16, 1917.)

In the previous paper[1] it has been shown that the cytolysis reaction in the case of the eggs of the sea urchin, *Strongylocentrotus purpuratus*, depends upon a chemical reaction of the first order. In these experiments the material was homogeneous; *viz.*, in each experiment the eggs were from one individual and were all mature and unfertilized. If one attempts to repeat the experiment with the eggs of starfish, the results fail to show a uniform progress of the reaction. This is apparently due to the fact that the starfish eggs from a given individual are not homogeneous. If a lot of these eggs are allowed to stand in the laboratory for a day and are then examined, very often sufficient change has occurred to render it possible to group them into three classes by optical and experimental means; *viz.*, (1) immature eggs with irregular outline and large nuclei, incapable of fertilization; (2) recently mature eggs, regular spheres, transparent yellowish brown in color, which may be fertilized and are therefore alive; (3) old mature eggs, black-brown in color, which cannot be fertilized and are therefore dead. These differences have been shown to be dependent upon oxidations, and the changes of maturation and disintegration can be inhibited by keeping the eggs in an atmosphere of hydrogen or by adding small quantities of KCN to the solution containing them.[2] Therefore, it seems reasonable to suppose that the optical and vital differences described are

[1] Moore, A. R., *J. Biol. Chem.*, 1916–17, xxviii, 475.

[2] Loeb, J., Artificial Parthenogenesis, Chicago, 1913, 27.

expressions of underlying chemical differences, and that these chemical differences are dependent upon the degree to which certain oxidation reactions have developed; in other words, upon the respective ages of the cells. These chemical differences due to age should become apparent in the cytolysis reaction; *i.e.*, eggs having the least natural resistance should cytolyze first, those having greatest natural resistance cytolyze last, and those of average resistance, comprising the great majority of the cells, cytolyze in approximately half the time. A reaction of this type does not progress at a constant rate, like a chemical reaction depending upon the fortuitous interaction of ions or molecules, but at the outset has a low velocity, when half completed has its maximum velocity, and in the last phase has a low velocity. Arrhenius[3] has suggested a simple method for determining whether reactions of cells proceed at the uniform rate of a chemical reaction or are dependent upon the age and natural resistance of the units taking part in the process. According to this method, if the $\log e\ N$ (N, number of cells surviving at the end of time, t) is plotted as ordinates against the time, t, as abscissæ, the graph is a straight line in the case of a chemical reaction, but a double curve graph in case the reaction depends upon the natural resistance of the cells. The following experiments show that the cytolysis reaction of starfish eggs follows the second type of reaction; *i.e.*, is dependent upon the specific chemical condition of each egg, which differs significantly with the age of the egg.

The experiments were carried out in the way previously described.[4] The material used consisted of the unfertilized eggs of the starfish, *Asterias forbesii*. After the initial lowering of the temperature to the extent of 0.2–0.3°C., due to the introduction of the charge of eggs into the cytolyzing chamber, the bath was kept within 0.1°C. of the temperature required.

The experiments recorded in Table I show the percentages of eggs remaining uncytolyzed at the end of each time interval. It is evident that the percentage of eggs cytolyzed each minute is not a constant percentage of the number remaining uncytolyzed. The graphs derived from the experimental data show that the

[3] Arrhenius, S., Quantitative Laws in Biological Chemistry, London 1915, 78.

[4] Moore, *Quart. J. Exp. Physiol.*, 1910, iii, 257.

TABLE I.

Time.	N	$\log_e N$.
Experiment 1. $T = 39°$.		
min.		
1	83	4.41
4	53	3.97
6	33	3.49
8	10	2.30
10	6	1.79
Experiment 2. $T = 38.7°$.		
1	77	4.34
2	52	3.95
4	47	3.85
6	18	2.89
8	16	2.77
Experiment 3. $T = 39°$.		
1	93	4.53
2	87	4.46
4	80	4.38
6	66	4.18
8	14	2.64
10	10	2.30
Experiment 4. $T = 39°$.		
1	94	4.54
2	82	4.40
4	63	4.14
6.25	15	2.70
8	7	1.94
10	4	1.38
Experiment 5. $T = 39°$.		
1	100	4.60
2	88	4.47
4	65	4.17
6	45	3.80
8	18	2.89
10	5	1.61
Experiment 6. $T = 39°$.		
1	78	4.35
2	71	4.26
4	36	3.58
6	22	3.09
8	20	2.99

reaction velocity is slow at first, most rapid when half completed, and slow again when the reaction nears completion.[5] In some cases the third phase does not appear. From these results it must be concluded that the rate of cytolysis of starfish eggs, *i.e.*, the time necessary at the given temperature for an egg to cytolyze, depends upon the chemical state of the egg, which in turn is determined by the age of the egg or the extent to which certain oxidations have taken place in it (Fig. 1).

The Cytolytic Effect of Treatment with the Alkaline Earth Chlorides.

It has been shown that treatment with an isosmotic solution of $SrCl_2$ greatly increases the rate of hot water cytolysis of the eggs of the sea urchin, *Strongylocentrotus purpuratus*.[6] In fact if the eggs of this form are allowed to remain in an isosmotic solution of $SrCl_2$ at room temperature, they form clear blebs at their peripheries and ultimately cytolyze. Solutions of $MgCl_2$, $CaCl_2$, and $BaCl_2$ bring about similar results but differ in certain characteristic ways in their action. Thus, in the solution of $MgCl_2$ the eggs swell and become clear; in the $CaCl_2$ they become clear with little if any swelling. In $BaCl_2$, as a rule, the cytoplasm remains optically unchanged, but a large bleb develops at the periphery, often equalling the egg in diameter; occasionally shadows form in this solution. KCl does not inhibit the cytolytic action of $MgCl_2$ and $CaCl_2$, nor are the two latter named salts mutually antagonistic. NaCl, however, when present in the ratio of 10 cc. of the M/2 solution to 90 cc. of a 3/8 M $CaCl_2$ or of a 3/8 M $MgCl_2$ solution completely inhibits the formation of blebs or of shadows.

In order to determine whether the chlorides of Mg, Ca, Sr, and Ba comparably affect the eggs of the starfish, *Asterias forbesii*, two types of experiments were carried out. First, the eggs were allowed to remain in the pure solutions of the chlorides of 3/8 M concentration for 12 to 24 hours. No definite cytolytic effects were observed. Second, the eggs, after being washed twice in the pure salt solution, were kept for 10 minutes in a third por-

[5] The writer is aware that this also describes in a general way the course of an autocatalytic monomolecular reaction, but if one tries to fit the data here given to the equation for such a reaction, it will be seen that the reaction proceeds much too rapidly at the outset for such an hypothesis to be applicable.

[6] Moore, *Arch. Entwcklngsmechn. Organ.*, 1913, xxxvii, 433.

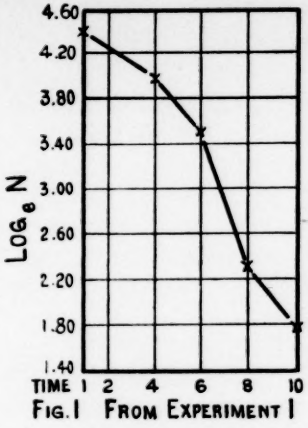

FIG. 1 FROM EXPERIMENT 1

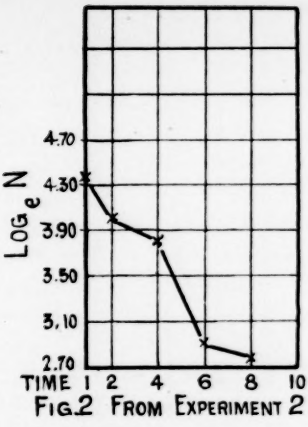

FIG. 2 FROM EXPERIMENT 2

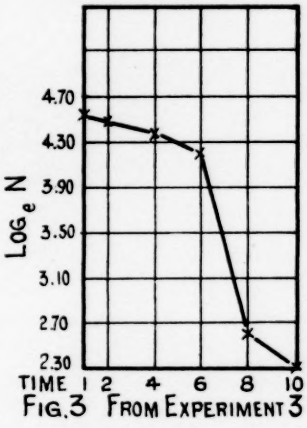

FIG. 3 FROM EXPERIMENT 3

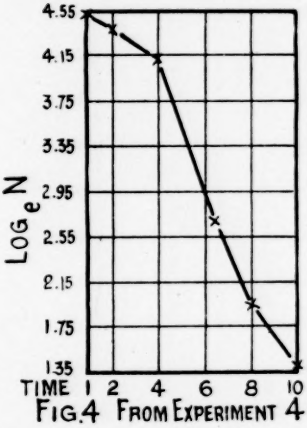

FIG. 4 FROM EXPERIMENT 4

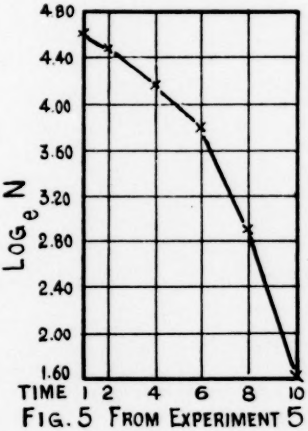

FIG. 5 FROM EXPERIMENT 5

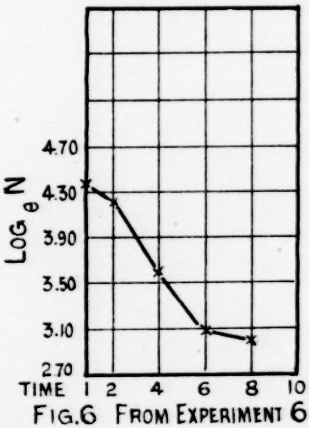

FIG. 6 FROM EXPERIMENT 6

FIG. 1.

tion of the solution and were then put into a cylinder of heated sea water kept at a constant temperature. The percentages of eggs cytolyzed were determined in the usual way. A parallel experiment with the untreated eggs of the same female was always run as a control. The experiments showed that in the eggs of the starfish the chlorides of Mg, Ca, Sr, and Ba not only fail to increase the rate of cytolysis but frequently retard it. The experiments in Table II illustrate this point. Since, in the case of sea urchin eggs, the chlorides of the alkaline earths exert a cytolytic action, the results of the experiments with starfish eggs indicate characteristic differences in the chemical constitution of the eggs of the two genera with respect to the phenomenon of cytolysis.

TABLE II.

Time.	Normal eggs cytolyzed.	Treated eggs cytolyzed.
	Experiment 1. $T = 39°$. $MgCl_2$.	
min.	*per cent*	*per cent*
2	12	5
4	35	
6	55	37
8	82	48
10	95	100
	Experiment 2. $T = 39°$. $CaCl_2$.	
	A.	
1	5	4
2	22	18
4	67	60
6	70	62
8	95	72
10	99	90
	B.	
1	100	5
2		20
4		80
6		90
8		95

TABLE II—*Concluded.*

Time	Normal eggs cytolyzed.	Treated eggs cytolyzed.
Experiment 3. $T = 39°$. $SrCl_2$.		
	A.	
1	1	0
2	2	0
4	2	2
6	40	
8		20
10	80	25
15	95	50
	B.	
1	23	22
2	48	29
4	53	64
6	82	78
8	84	80
Experiment 4. $T = 39°$. $BaCl_2$.		
1	9	1
2	30	
4	48	5
6	80	
8	98	
10	100	10

THE SUPPLEMENTARY DIETARY RELATIONSHIP BETWEEN LEAF AND SEED AS CONTRASTED WITH COMBINATIONS OF SEED WITH SEED.*

By E. V. McCOLLUM, N. SIMMONDS, and W. PITZ.

(From the Laboratory of Agricultural Chemistry of the Wisconsin Experiment Station, Madison.)

(Received for publication, March 26, 1917.)

In previous papers we have described the nature of the dietary deficiencies of some of the most important seeds which serve as food for man and animals; *viz.*, wheat,[1] maize,[2] rice[3] and oat kernels,[4] and the white bean.[5] With the exception of polished rice and the bean these seeds closely resemble each other in their dietary properties: (*a*) the proteins being of relatively poor quality in that certain essential amino-acids are furnished by them in amounts below the optimum; (*b*) the content and composition of the inorganic portion of each seed are of a character which cannot induce normal growth, and must be corrected by certain salt additions; and (*c*) the content of the fat-soluble A is inadequate to supply the needs of the growing animal over a prolonged period. Unpolished rice resembles closely the other grains mentioned above, but polished rice, which has lost both its bran layer and its germ in the process of polishing, lacks, in addition to the deficiencies mentioned above, the second unidentified dietary

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Hart, E. B., McCollum, E. V., Steenbock, H., and Humphrey, G. C., *Wisconsin Agric. Exp. Station Research Bull. 17*, 1911. Hart and McCollum, *J. Biol. Chem.*, 1914, xix, 373. Hart, E. B., Miller, W. S., and McCollum, E. V., *ibid.*, 1916, xxv, 239. McCollum, E. V., Simmonds, N., and Pitz, W., *ibid.*, 1916–17, xxviii, 211.

[2] Hart and McCollum, *J. Biol. Chem.*, 1914, xix, 373. McCollum, Simmonds, and Pitz, *ibid.*, 1916–17, xxviii, 153.

[3] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 181.

[4] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1917, xxix, 341.

[5] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1917, xxix, 521.

factor, water-soluble B. The ordinary bean differs from the other seeds mentioned particularly in the poor quality of its proteins. These contain but little of some one or more essential amino-acids and it becomes imperative that beans should be combined with such other natural foods as furnish proteins which supplement these deficiencies and therefore enhance their value.

In the present paper we present certain further data which point clearly to the general lines on which successful nutrition is to be attained, when the diet is derived solely from vegetable sources. The discussion will be limited to the results obtained with combinations of seeds from several sources, and of seeds with the alfalfa leaf. We reserve for future discussion the possibilities of obtaining normal nutrition during growth and reproduction with simple combinations of tubers with grains and of fruit juices with grains.

Our experience over a decade with both rats and swine has convinced us that the nutritive requirements for these two species are essentially the same. Neither species can grow satisfactorily when restricted to one of the cereal grains, and both respond in about the same way with growth and reproduction, to specific modifications of the diet when thus restricted as to source.

It was somewhat of a surprise to us to find that we were not able to make up a ration derived solely from the seeds of plants which would serve to support normal nutrition during the growing period, even though two to five seeds of widely different varieties were employed. Results of a very different character are secured when simple combinations of leaf and seed are fed as monotonous diets. The interpretation of the curves of growth in the charts presented in this paper bring out the following points:

1. We have failed to secure any appreciable amount of growth with the following mixtures of seeds when salt-free water was supplied; (*a*) maize 90, flaxseed oil meal 10, Chart 1; (*b*) wheat 20, maize 20, rolled oats 20, hemp seed 20, millet seed 20, Chart 4; (*c*) wheat 25, maize 25, rolled oats 25, hemp seed 25, Chart 3; (*d*) wheat 33, maize 33, rolled oats 33, Chart 2. These rations represent two distinct types: (*a*) and (*b*) serve to promote good growth if suitable inorganic salt additions are made, but not otherwise (Charts 1 and 4). This means that the mixtures sup-

ply enough of both the unidentified dietary factors fat-soluble A and water-soluble B, as well as the long recognized dietary factors, proteins of adequate quality, and assimilable energy as carbohydrate and fat. When considered in the light of our former experience with maize, wheat, and oats, all of which have been shown to carry an inadequate amount of the fat-soluble A, it becomes evident that the flaxseed meal and millet seed differ from maize, wheat, and oats in containing a much greater amount of this dietary factor. This conclusion is further supported by the records of rats fed (*c*) (Chart 3). After a period of suspended growth they failed to respond so well with growth when a salt mixture was added as did those whose diets contained either flaxseed or millet seed (Charts 1 and 4). Hemp seed appears to be somewhat richer in the fat-soluble A than are wheat, oats, and maize. Ration (*d*) does not support growth, and Period 2 in the record of Lot 714 (Chart 2) shows clearly that it is not solely the shortage of the fat-soluble A which is responsible. Our experimental work does not yet include the feeding of wheat, oat, and maize mixtures with salts alone or purified protein alone, but in view of the remarkable degree to which salt additions induce growth on mixtures of these seeds with hemp seed and millet seed (Charts 3 and 4) there is little doubt that this is one of the limiting factors in the case of Lot 714. In other words, with wheat, oats, and maize in equal proportions the data available indicate that both salts and fat-soluble A must be added before growth can take place.

The growth records shown in Charts 1 to 4 make possible the generalization that *it is difficult if not impossible to obtain even a moderate amount of growth over an extended period on a diet restricted to the seeds of plants.* It is evident that satisfactory protein mixtures can be had from seed mixtures, and from the results shown in Charts 1 and 4 it is further evident that certain seeds as flaxseed and millet seed contain the fat-soluble A in fairly liberal amounts. Since the water-soluble B is everywhere abundant in the seeds the cause of failure to secure growth on seed mixtures is seen to lie in the amount and character of the inorganic moiety.[6] An in-

[6] Unpublished data in our records indicate that the mixed proteins of wheat, oat, and maize fed at this plane of protein intake (10 to 11 per cent) are of satisfactory character to support fairly good growth when the rations are not otherwise deficient.

spection of the literature relating to the content of inorganic elements in various classes of natural foods as determined in recent times by satisfactory methods reveals at a glance the supplementary relationships among the different classes of vegetable foods in inorganic elements. Of the seven most important seeds from the standpoint of human nutrition and animal production, only cottonseed and flaxseed contain a high total inorganic content and in both cases the ash is very poor in three important elements, sodium, calcium, and chlorine. Since a pronounced deficiency of these elements is characteristic of all other seeds as well, no combinations of seeds will supply these elements in satisfactory amounts. Since spring and well waters in certain districts contain both sodium and calcium in greatly varying amounts it would be expected that animals may secure the necessary inorganic supplements through the drinking water in some localities which would enable them to grow on a ration restricted to seeds, whereas in other parts of the world they could not do so.

But few reliable data are available showing the variation of the inorganic content of seeds as influenced by the character of the soil. It is a well known principle of physiology that plants have but limited selective capacity with reference to minerals in solution in the soil water. Forbes and his coworkers[7] made an extensive investigation of the inorganic elements of blue grass from different types of soils and found that at the same stage of growth samples from different localities showed variation between 4.80 and 8.66 per cent of ash, calcium varied between 0.135 and 0.424 per cent, potassium between 1.41 and 2.85 per cent, and phosphorus between 0.164 and 0.403 per cent.

Ames[8] states that the composition of wheat (grain) grown on the unfertilized plots of two soils, containing different amounts of phosphorus, potassium, and nitrogen is in accordance with the composition of these soils, and that the proportion of these elements in the wheat plant is increased by the addition of these elements to the soil. Under natural conditions distinct differences will doubtless be observed in the capacity to grow of animals

[7] Forbes, E. B., Whittier, A. C., and Collison, R. C., *Ohio Agric. Exp. Station Bull. 22*, 1910.

[8] Ames, J. W., *Ohio Agric. Exp. Station Bull. 221*, 1910. Ames, J. W., Boltz, G. E., and Stenius, J. A., *ibid., 243*, 1912.

*Inorganic Constituents of Vegetable Foodstuffs.**

	Ash.	K	Na	Ca	Mg	P	Cl
			Leaf.				
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Alfalfa hay	7.38	1.64	0.1	2.14	0.22	0.34	0.29
Rape green	8.08	2.23	0.23	1.26	0.18	0.40	0.61
Red clover	6.86	1.84	0.10	1.71	0.45	0.29	0.26
Rhubarb	14.49	7.14	0.55	1.04	—	0.93	0.77
Spinach	16.48	2.27	4.32	1.40	0.63	0.74	1.04
Cabbage heart	10.85	2.51	0.80	1.66	0.23	0.71	0.85
			Seed.				
Rice hulled	0.39	0.07	0.016	0.009	0.026	0.09	0.0004
Maize	1.55	0.32	0.05	0.01	0.12	0.33	0.045
Wheat	2.08	0.50	0.02	0.04	0.14	0.40	0.06
Oat meal	1.83	0.36	0.06	0.01	0.09	0.38	0.10
Beans	3.63	1.25	0.03	0.13	0.16	0.61	0.06
Cottonseed meal	7.48	1.85	—	0.23	0.68	1.5	0.003
Linseed meal	5.84	1.18	0.07	0.35	0.55	0.80	0.04
			Tuber, root.				
Potato	3.79	1.90	0.08	0.07	0.11	0.28	0.13
Sweet potato	3.07	1.28	0.15	0.22	0.06	0.14	0.39
Beet	5.97	0.84	2.16	0.25	0.01	0.25	0.29
Turnip	8.01	3.02	0.58	0.60	0.18	0.44	0.40
			Fruit.				
Oranges	3.08	0.93	0.31	0.54	0.15	0.15	0.07
Apples	1.44	0.43	0.28	0.04	0.07	0.08	—
Plums	2.08	1.20	0.03	0.06	0.06	0.12	0.007
Raisins	2.86	1.10	0.16	0.10	0.10	0.22	0.14
Figs	2.92	1.35	0.05	0.23	0.10	0.16	0.06

* Selected from the paper by E. B. Forbes, *Ohio Agric. Exp. Station Bull. 207*, 1909.

confined to a diet of seeds but permitted access to the soil and furnished ground or drainage water, because of the variable factors mentioned above. It is a matter of great importance, however, that we should fully appreciate the peculiar dietary properties of the different classes of vegetable foods and bear in mind

the importance of making suitable combinations which will approximate the optimum in content and proportions among the inorganic elements.

The degree to which the inorganic content of certain mixtures of seed and leaf supports growth will be seen from an inspection of Charts 5 to 14. It should be kept in mind, however, that a complete explanation is not available of the cause of the relatively poor nutrition of many of the animals fed the rations described in this paper. Not until those food mixtures which fail to nourish young animals properly are fed supplemented with certain single and multiple additions of purified food substances can such explanations be made. Where little or no growth was observed certain dietary factors other than protein, inorganic constituents, or unidentified factors A and B may be the cause. These are to be excluded by the results of suitably planned experiments.

A second generalization of fundamental importance in nutrition is now possible.[9] *The leaf is distinctly different from the seed in its dietary properties in two respects: its total inorganic content is very high, and it is especially rich in both sodium and calcium, both of which are deficient in the seeds generally.* In addition the leaf of the plant is several times richer in fat-soluble A than are the wheat, oat, and maize kernels. Certain seeds approximate the value of the leaf in this substance. Hemp seed is distinctly better than those just named, but flaxseed and millet seed are still richer than hemp seed and may readily be incorporated in the diet in amount sufficient to meet the needs of an animal for the fat-soluble A during growth (Charts 1 and 4).

It is interesting to note that the content of the fat-soluble A is highest in those seeds which are smallest (flax and millet). It seems not unlikely that this may in some measure be related to the relatively large proportion of germ as compared with endosperm in such seeds. The endosperm is in great part to be likened to a mixture of purified proteins, carbohydrates, and fats, while the germ is relatively rich in functioning plant cells as well as fats, carbohydrates, etc. The great differences in the amount of stored food material contained in the germ in various seeds ren-

[9] McCollum, Simmonds, and Pitz, *Am. J. Physiol.*, 1916, xli, 333.

der it impossible to make such a comparison very accurate. It seems reasonable to suppose that the differences in the dietary properties of the entire seed as contrasted with the embryo,[10] and of polished rice as contrasted with hulled rice, may lie in part in the association of the unidentified dietary A and B with the functioning cell. The same reasoning may be applied to account for the exceptional richness of the leaf in these two dietary principles. The leaf is the seat of great synthetic activity and consists relatively of a large amount of functioning cells associated with those substances which may be considered as the equivalent of purified protein, carbohydrate, and fats.

We have pointed out in a previous paper[11] that the isolated plant fats as obtained either by extraction with a fat solvent or by hot pressing do not contain the fat-soluble A. Suitably planned feeding experiments have shown that it is the fat-free residue of the seed which still contains the content of this substance which was originally present in the seed. We have demonstrated this only in the case of the maize kernel[2, 11] and the flaxseed (Chart 1, Lot 722), but the experiments of Richardson and Green[12] indicate that cottonseed flour still contains a fair amount of this dietary factor. We have shown that isolated cottonseed oil is without the peculiar growth-promoting property which it would show if it contained this substance.[11]

[10] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916, xxv, 105.
[11] McCollum, Simmonds, and Pitz, *Am. J. Physiol.*, 1916, xli, 361.
[12] Richardson, A. E., and Green, H. S., *J. Biol. Chem.*, 1916, xxv, 307.

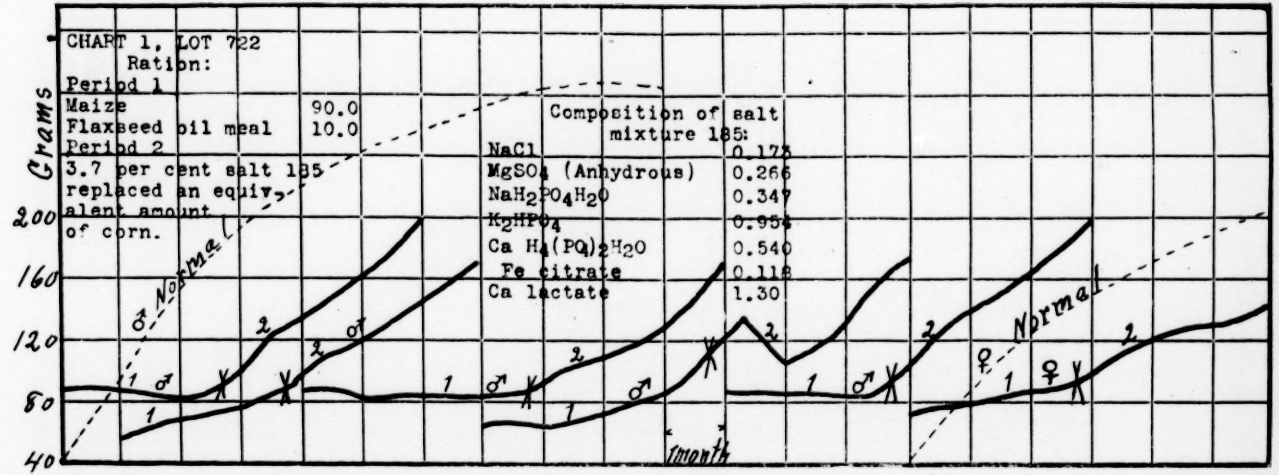

CHART 1. Lot 722 illustrates the failure of young rats to grow on a diet consisting of 90 per cent ground maize and 10 per cent flaxseed oil meal. The sole reason for the stunting lay in the unsatisfactory character of the inorganic content of the food mixture. After 3 to 4 months of suspended growth the animals began to increase in body weight at nearly the normal rate when 3.7 per cent of a suitably constituted salt mixture was added. The fact that growth took place indicates that the protein mixture was of satisfactory character.[13] Salt-free water was supplied in all the experiments.

[13] McCollum, *J. Biol. Chem.*, 1914, xix, 323.

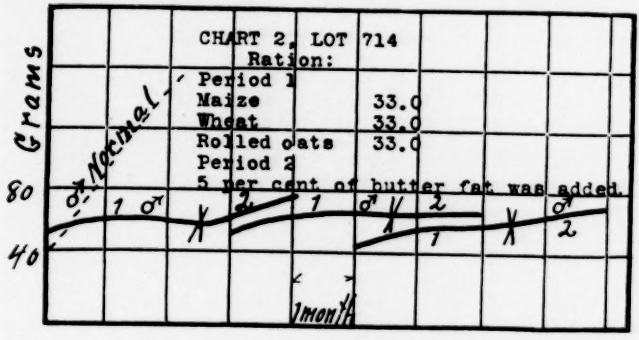

CHART 2. Lot 714 illustrates the failure of animals to grow when given a mixture of wheat, oats, and maize in equal proportions with distilled water. Two factors operate in contributing to the inadequacy of this diet for growth; *viz.*, there is an unsatisfactory inorganic content and a shortage of the fat-soluble A. In Period 2 the latter factor was added in butter fat, but there could be no response with growth without the deficiencies of the inorganic portion of the food mixture being made good. The mineral deficiencies of each of these grains have been demonstrated elsewhere.[1, 2, 4]

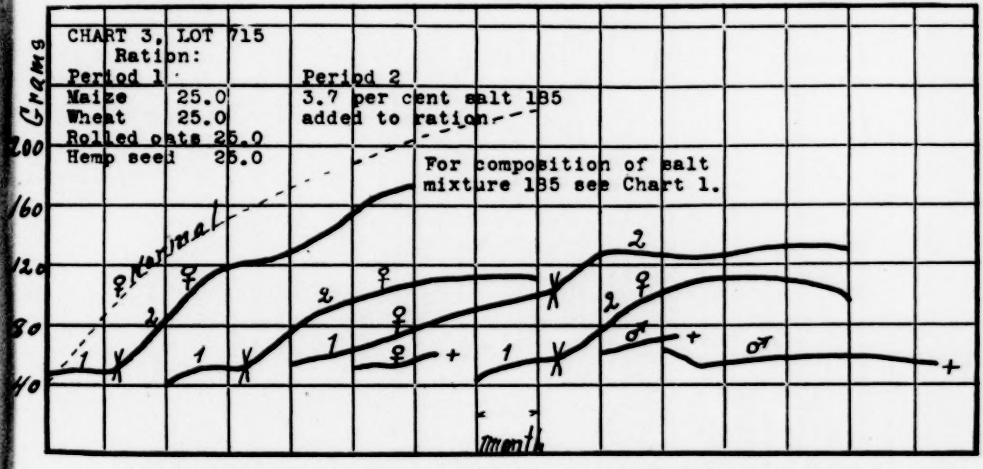

CHART 3. Lot 715. Practically no growth can be secured with even four seeds, as the above records show. There was but a fair response with growth when a suitable salt mixture was added after a period of suspension. The reason for this lies in the low content of the unidentified fat-soluble A in these seeds. Hemp seed is considerably richer than the other seeds in the mixture in this substance so the rats were enabled to grow at a rate below the normal. If both salt and butter fat additions had been made simultaneously, growth would have been much more rapid.

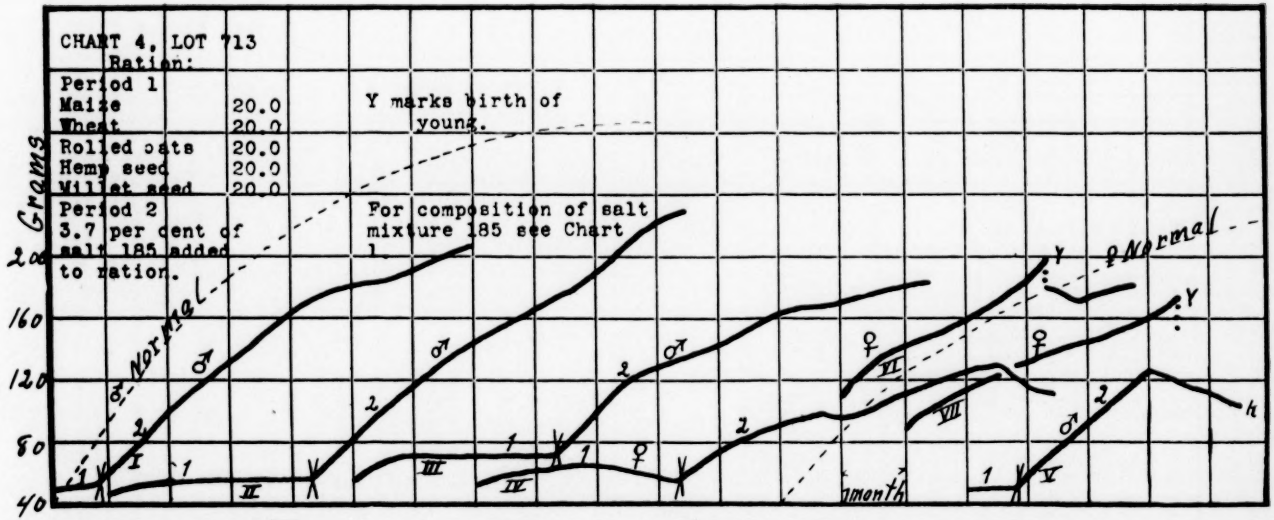

CHART 4. Lot 713. Even five seeds in wholesome condition when mixed in equal proportions cannot support growth when fed without the possibility of an accessory salt supply, as might happen if certain well or spring waters were taken. The sole reason for the failure of these animals to grow is found in the fact that the seeds generally furnish but an inadequate amount of the inorganic elements essential for normal metabolism, and all are especially poor in the elements sodium and calcium. In Period 2 the addition of 3.7 per cent of Salt Mixture 185 induced a prompt resumption of growth. The resumption of growth was possible because millet seed like flaxseed contains a fairly liberal amount of the fat-soluble A. Hemp seed contains more than either wheat, oats, or maize, all of which are distinctly poor in this dietary factor. Compare with Charts 2 and 3. Rat VI, which was fed this seed mixture with salt additions from the beginning, has successfully weaned five young. These weighed collectively 412 gm. at 72 days of age.

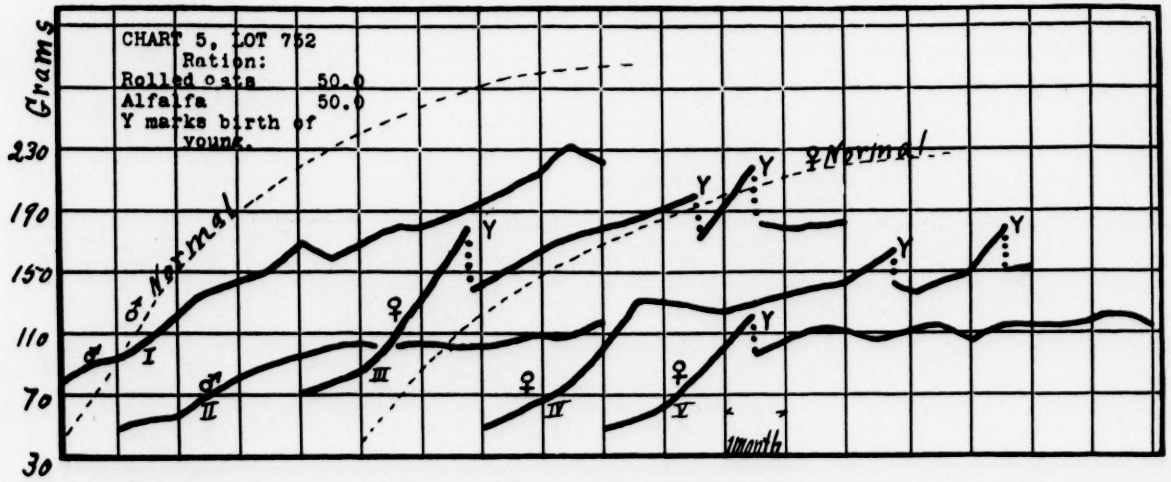

CHART 5. Lot 752. These curves illustrate the remarkable difference in the growth-promoting properties of a food mixture of the oat kernel with the alfalfa leaf in equal proportions as contrasted with the several mixtures of seeds alone described in Charts 1 to 4. Of the five litters of young born (thirty young) three young were successfully weaned. Four litters were eaten by the mothers within a few days. Rat III kept her last litter of six to the age of 14 days. They were then scaly and scrawny and weighed but 12.4 gm. each. Three have reached the age of 41 days and weigh but 37 gm. each. The normal for this age is about 60 gm. On becoming able to eat of the mother's ration they became bright and active. The alfalfa content of this ration is above the optimum (Chart 6).

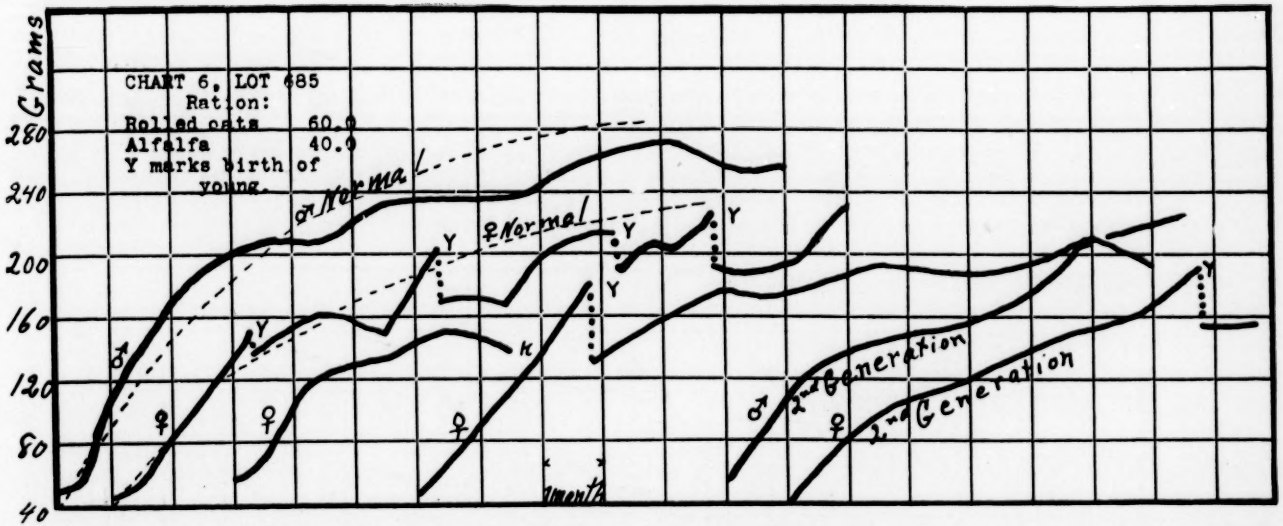

CHART 6. Lot 685. Rolled oats 60 and alfalfa leaf 40 per cent make a surprisingly good ration for the rat, both for growth and for rearing of the young. This ration is much better than is a mixture of oats and alfalfa leaf in equal amounts (Chart 5). All the records of our numerous trials of alfalfa with other food materials indicate that it should never contribute more than 40 per cent of the food mixture. Oats and alfalfa form a better monotonous ration than does alfalfa with any other seed we have studied (compare Charts 5 to 14). The proportions furnished to these animals, 40 of alfalfa to 60 of oats, are the most satisfactory for the promotion of well-being. Of the six litters (thirty-three young) sixteen young were successfully weaned but were somewhat undersized. Two of these have grown at somewhat below the normal rate to the age of 6 months. The nutrition of the animals in Lot 685 is below the optimum, but this can be seen only in the high mortality of the young, and in the fact that there is a tendency for the rats to remain undersized. This is especially true of the second generation confined to this ration.

CHART 7. Lot 753. This chart confirms the statement made in discussing Chart 6, that rolled oats and alfalfa leaf in the proportions 60 to 40 form the most satisfactory mixture of these two substances (compare Charts 5 to 8). With a mixture of oats 70 and alfalfa 30 nearly complete growth was secured in half the animals, the rest suffering partial stunting. Of three females in this group one has produced no young and the other two but single litters of six and three. Five of these young were successfully weaned.

Lot 872 shows the rate of growth during the first 3 months on a diet of 80 per cent of oats and 20 of alfalfa.

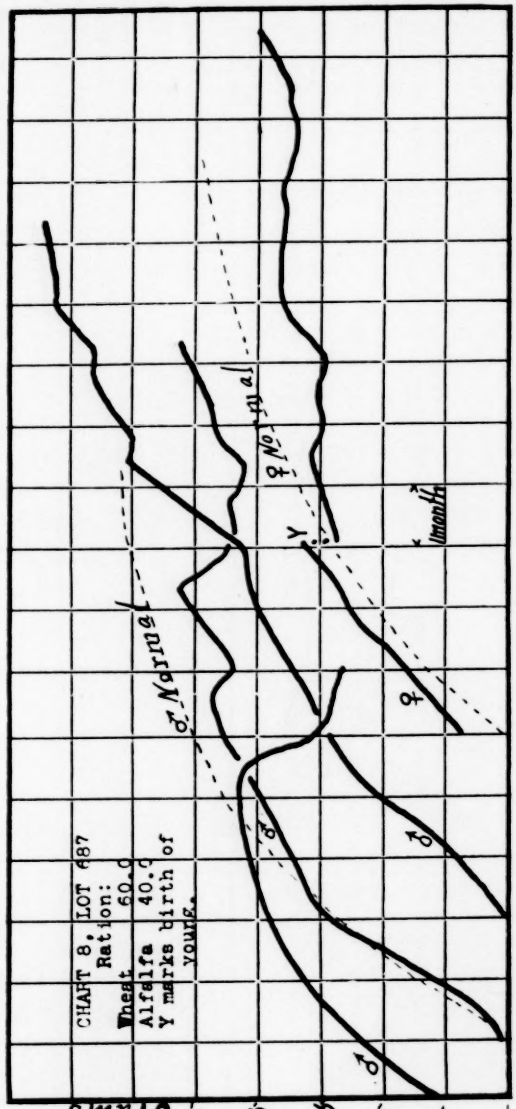

CHART 8. Lot 687 illustrates the irregularity in the course of the growth curves, but all approached the normal curves very closely. This lot contained but one female and she has produced but one litter of young, all dead when born. It is evident that 60 of wheat with 40 of alfalfa is a distinctly inferior ration as compared with the same proportions of oats and alfalfa (Chart 6).

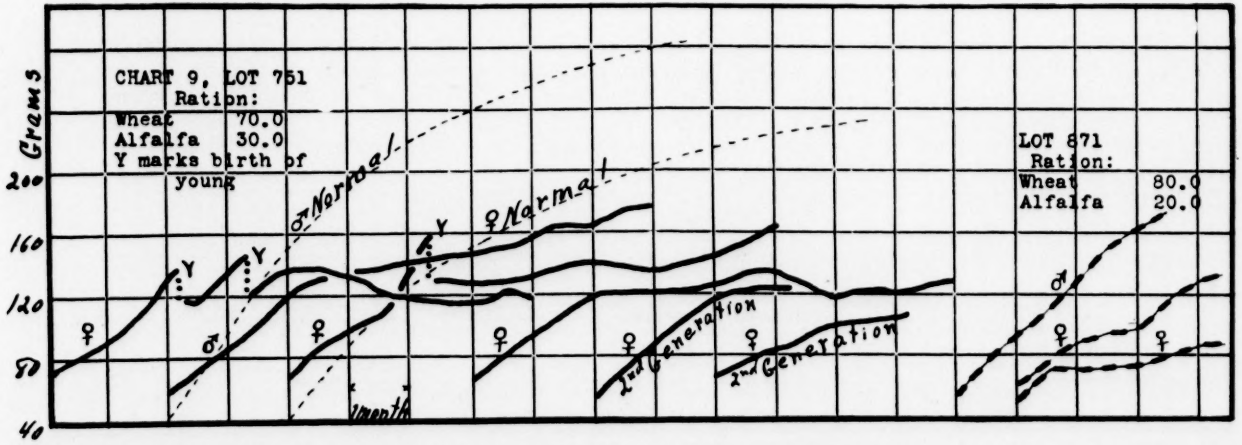

CHART 9. Lot 751 shows the pronounced stunting suffered by rats fed 70 per cent of wheat and 30 per cent of alfalfa. One of the three litters of young born was successfully weaned. These young are failing to grow on this diet. The inferiority of wheat and alfalfa combinations as contrasted with similar mixtures of oats and alfalfa for growth is very striking (compare with Chart 7).

Lot 871. Three of the four rats are markedly stunted and the fourth is growing at a rate slower than the normal on wheat 80 and alfalfa 20 per cent. It will be necessary to make a special series of feeding trials with each of these wheat and alfalfa mixtures supplemented with single and multiple additions of purified foodstuffs in order to learn the specific reason for their unsatisfactory nutrition.

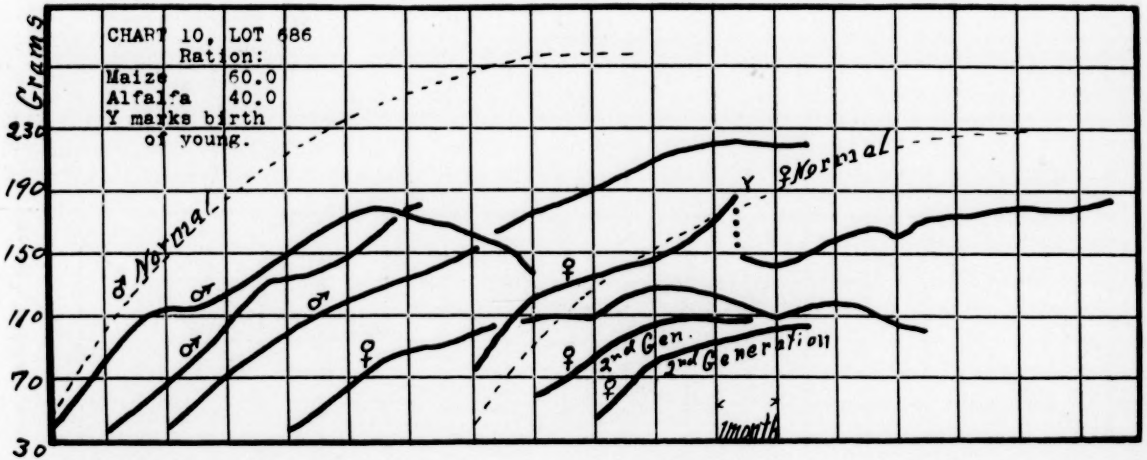

CHART 10. Lot 686 shows distinctly poorer nutrition of animals fed maize 60 and alfalfa 40 than was secured with oats and alfalfa in similar proportions (Chart 6). The growth is, however, much better than we have ever been able to secure with diets restricted to the seeds of plants (Charts 1 to 4). The single litter of young which was secured from the two females of this lot was successfully weaned. The second generation confined to this food mixture is failing to grow. These animals all show distinct signs of old age at 11 months.

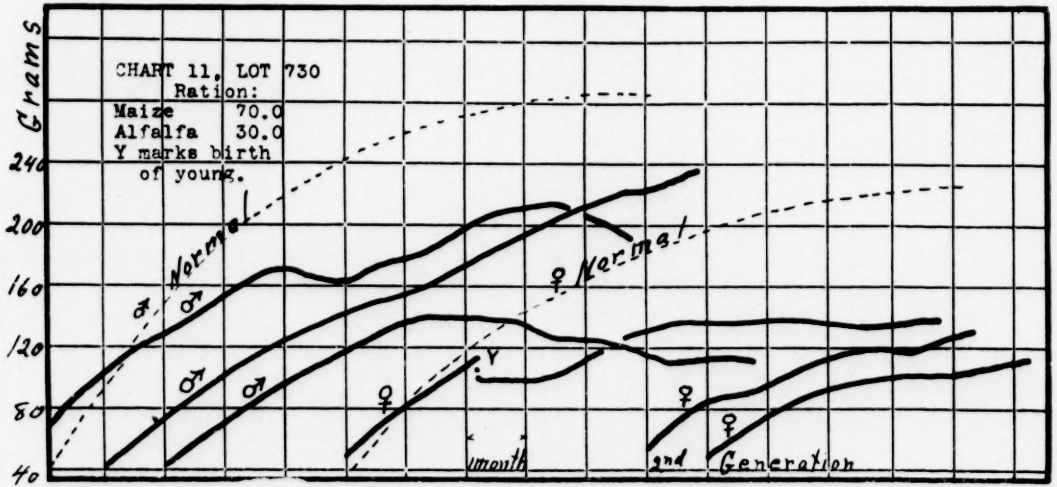

CHART 11. Lot 730. This chart when compared with Chart 10, Lot 686, and with Chart 12, Lot 729, seems to indicate definitely that within certain limits lower planes of alfalfa (10 to 20 per cent) are more successful as supplements to the maize kernel than are higher planes (30 to 40 per cent) such as are most successful with oats (Charts 6 and 7). This is further supported by Chart 10. The reason why the growth and well-being of animals fed maize and alfalfa fall distinctly below the normal must be found by systematic inquiry as described under Chart 9. These records are presented here for the sake of emphasizing the superiority of rations composed of leaf and seed over those made by combining seed with seed (Charts 1 to 4).

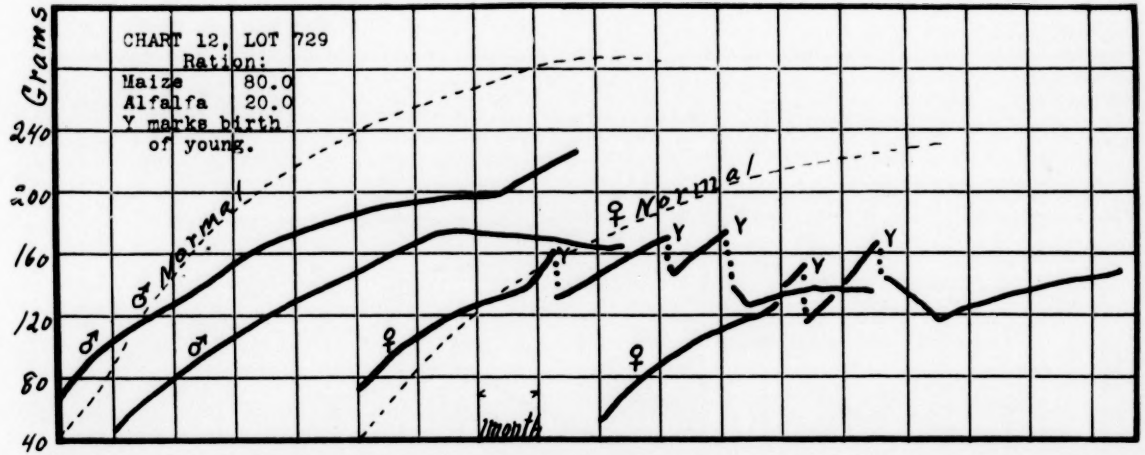

CHART 12. Lot 729. The rats in this group receiving 20 per cent of alfalfa with 80 per cent of maize grew noticeably better than did those receiving higher planes of alfalfa leaf (Charts 10 and 11). Although the mortality of the young was high (eighteen young born and four weaned), the young which survived to an age when they were independent of the mother's milk supply and could eat the mother's ration greatly improved thereafter. Although somewhat undersized, these rats appear to be well nourished. It is hardly probable that growth curves of this character can ever be secured with any combinations of seeds, however complex the mixture, provided salt-free water be given and access to the soil be denied.

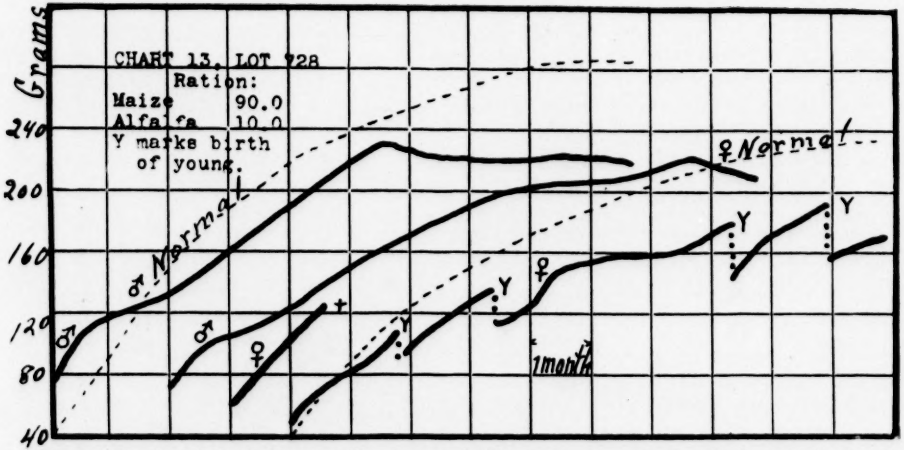

CHART 13. Lot 728. The growth curves of this group are on the whole a little better than those of any other group fed maize and alfalfa. One female died from an unknown cause early in life. The remaining one has produced four litters of young; none of the first three litters were kept alive beyond a few days. Of the fourth litter of eight young, three were reared. These, continued on the ration, weighed 75 gm. at 27 days of age. If fed 100 per cent maize kernel, not the slightest amount of growth would ever be secured. The supplementary relationship of the 10 per cent of alfalfa in this ration is of profound importance to the animal.

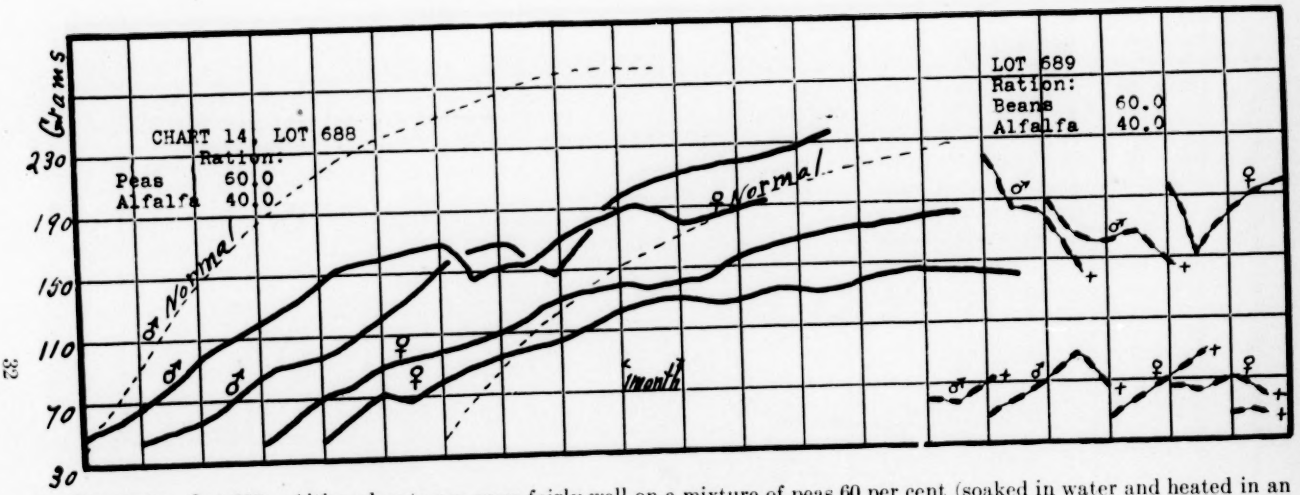

CHART 14. Lot 688. Although rats can grow fairly well on a mixture of peas 60 per cent (soaked in water and heated in an autoclave for 1¼ hours at 15 pounds' pressure) and alfalfa leaf 40, no young have been secured from this group, although it contained two females.

Lot 689. In marked contrast to the growth secured in Lot 688 with 60 per cent of peas and 40 of alfalfa, no growth has been observed in rats fed 60 per cent of beans and 40 per cent of alfalfa. The mortality of animals in this group has been very high in the first few weeks of feeding. A special investigation will be necessary to reveal the reason for this pronounced failure. These results emphasize what has not been hitherto appreciated; *viz.*, that the pea and the bean are very different from the dietary standpoint.

THE PROTEINS OF THE PEANUT, ARACHIS HYPOGÆA.

II. THE DISTRIBUTION OF THE BASIC NITROGEN IN THE GLOBULINS ARACHIN AND CONARACHIN.*

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(Received for publication, March 14, 1917.)

The culture of peanuts in the United States has increased rapidly during the last ten years. During the year 1916 a large number of mills which were formerly engaged in pressing cottonseed to obtain oil and press cake were unable to secure a sufficient supply of cottonseed and are now pressing peanuts. Thus, there has been produced a large supply of peanut oil and peanut press cake. The oil, which compares favorably with olive oil for culinary purposes and is also used for making oleomargarine, has found a ready market and was quoted February, 1917, at $1.05 per gallon. The press cake is ground to a meal and is readily finding favor as a stock food. It is quoted at $35 per ton. When this meal is made from whole peanuts it contains about 28 per cent of protein, while meal made from shelled peanuts contains about 45 per cent of protein (N x 6.25).

In a recent paper (1) from this laboratory it was shown that the globulins of the peanut yield a relatively high percentage of basic nitrogen. Arachin, which is the chief protein of the peanut, contains 4.96, and conarachin 6.55 per cent. These values were obtained by the Hausmann method in which no correction is made for the solubility of the phosphotungstates of the bases and are therefore somewhat lower than the figures obtained by the Van Slyke method in which such a correction is applied. These proteins have now been analyzed by the Van Slyke method to ascertain the percentage of the basic amino-acids. The dis-

* Published by permission of the Secretary of Agriculture.

tribution of nitrogen previously reported from analyses by the Hausmann method are given in the following table (2).

N	Arachin.	Conarachin.
Amide	2.03	2.07
Humin	0.22	0.22
Basic	4.96	6.55
Non-basic	11.07	9.40
Total	18.28	18.24

The results of the analyses by Van Slyke's method show that both arachin and conarachin contain arginine, histidine, lysine, and cystine. Both proteins also give a strong qualitative test for tryptophane.

Percentage of Basic Amino-Acids in the Globulins of the Peanut.

	Arachin.	Conarachin.
Arginine	13.51	14.60
Histidine	1.88	1.83
Lysine	4.98	6.04
Cystine	0.85	1.07

The figures for cystine are undoubtedly too low, as they represent only the cystine which escaped destruction during the hydrolysis of the proteins with hydrochloric acid.

The most important fact brought out by these analyses is the high content of lysine in arachin and conarachin. For the sake of comparison the percentages of lysine in the proteins of some common seeds and in muscle from some animals are also given in the following tables.

The figures marked by (§) were obtained by the Van Slyke method and, therefore, probably represent the maximum percentage of lysine obtainable. Those not so marked were obtained by Kossel's absolute method and may be somewhat too low. It is seen, however, that the percentages of lysine in arachin and conarachin of the peanut are relatively high, and, indeed, approach the lysine content of muscle substance of different animals. It will also be noted that no lysine has been found in zein

and that the maximum percentage of lysine obtained from gliadin is only 1.21 per cent.

Osborne and Mendel (3) and other workers have shown that lysine is essential to the growth of animals. Nutrition experiments indicate (4) that the animal organism cannot synthesize lysine which must, therefore, be provided in suitable quantity in the food to insure normal growth. Since the muscle substance of animals contains about 7 per cent of lysine, foods deficient in this essential amino-acid should be supplemented by the

Lysine Content of Some Vegetable Proteins.

Protein.	Source.	Percentage.
Zein.	Maize.	0.00*
Gliadin.	Wheat.	§1.21**
Legumin.	Pea.	4.29†
Phaseolin.	Kidney bean.	4.58‡
Arachin.	Peanut.	§4.98
Conarachin.	"	§6.04

* Osborne, T. B., and Jones, D. B., *Am. J. Physiol.*, 1910, xxvi, 227.

** Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, xxii, 259.

† Osborne, T. B., and Clapp, S. H., *J. Biol. Chem.*, 1907, iii, 219.

‡ Osborne, T. B., *Ergebn. Physiol.*, 1910, x, 116.

*Lysine in Muscle Substance of Different Animals.**

Scallop (*Pecten irradians*)	5.77
Halibut (*Hippoglossus vulgaris*)	7.45
Chicken	7.24
Ox	7.59

* Osborne and Jones, *Am. J. Physiol.*, 1909, xxiv, 438.

addition of other foods which contain a high percentage of lysine. Wheat and corn, both of which contain but little lysine, should therefore prove more efficient diets if supplemented by some food of high lysine content. Peanut meal appears to be well adapted to this purpose. From a nutritive standpoint, it is one of our cheapest foods and seems to possess no objectionable properties. Animals fed on it thrive and increase rapidly in weight (5). It therefore seems probable that corn and wheat could be much better utilized and a considerable saving in the cost of feeding effected by supplementing these cereals with peanut meal.

Peanuts may be used in many ways for human consumption. The Laboratory of Plant Chemistry of the Bureau of Chemistry has found that a mixture of 75 per cent of wheat flour and 25 per cent of peanut flour makes excellent bread. Such a bread is higher in protein content and contains much more lysine than bread made from wheat alone.

Investigations on the proteins of the peanut will be continued.

EXPERIMENTAL.

Analysis of Arachin.—Duplicate samples of 3 gm. of arachin, each equivalent to 2.7210 gm. of ash and moisture-free protein, were hydrolyzed by boiling with 100 cc. of 20 per cent hydrochloric acid for 24 hours. The protein contained 18.28 per cent of nitrogen, equivalent to 0.4974 gm. in each of the samples analyzed. The phosphotungstates of the bases were decomposed by the amyl alcohol-ether method. The following results are corrected for the solubilities of the phosphotungstates of the bases.

Analysis of Arachin. Van Slyke Method.

Total Nitrogen Corrected for Solubility of Bases.

	I.	II.	I.	II.	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N	0.0581	0.0594	11.68	11.94	11.81
Humin N adsorbed by lime	0.0028	0.0029	0.56	0.59	0.57
Humin N in amyl alcohol extract	0.0035	0.0007	0.71	0.15	0.43
Cystine N	0.0037	0.0036	0.75	0.73	0.74
Arginine N	0.1182	0.1183	23.76	23.78	23.77
Histidine N	0.0112	0.0166	2.24	3.33	2.78
Lysine N	0.0257	0.0262	5.17	5.28	5.22
Amino N of filtrate	0.2640	0.2662	53.08	53.52	53.30
Non-amino N of filtrate	0.0076	0.0089	1.52	1.79	1.65
Total N regained	0.4948	0.5028	99.47	101.11	100.27

Basic Amino-Acids in Arachin.

	I.	II.	Average.
	per cent	*per cent*	*per cent*
Arginine	13.50	13.51	13.51
Histidine	1.51	2.25	1.88
Lysine	4.93	5.03	4.98

Analysis of Conarachin.—Duplicate samples of 3 gm. of conarachin, each equivalent to 2.6967 gm. of ash and moisture-free protein, were hydrolyzed by boiling with 100 cc. of 20 per cent hydrochloric acid for 24 hours. The protein contained 18.23 per cent of nitrogen, equivalent to 0.4916 gm. in each of the samples analyzed. Ether and amyl alcohol were used to decompose the phosphotungstates of the bases. The following results are corrected for the solubilities of the bases:

Analysis of Conarachin. Van Slyke Method.
Total Nitrogen Corrected for Solubility of Bases.

	I.	II.	I.	II.	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N	0.0548	0.0542	11.14	11.02	11.08
Humin N adsorbed by lime	0.0031	0.0033	0.64	0.67	0.65
Humin N in amyl alcohol extract	0.0007	0.0006	0.13	0.12	0.13
Cystine N	0.0046	0.0028	0.93	0.58	0.75
Arginine N	0.1247	0.1288	25.37	26.19	25.78
Histidine N	0.0131	0.0136	2.67	2.78	2.72
Lysine N	0.0320	0.0305	6.51	6.20	6.35
Amino N of filtrate	0.2486	0.2452	50.57	49.88	50.23
Non-amino N of filtrate	0.0089	0.0102	1.81	2.08	1.94
Total N regained	0.4905	0.4892	99.77	99.52	99.63

Basic Amino-Acids in Conarachin.

	I.	II.	Average.
	per cent	*per cent*	*per cent*
Arginine	14.37	14.84	14.60
Histidine	1.79	1.87	1.83
Lysine	6.20	5.89	6.04

SUMMARY.

1. The globulins of the peanut have been analyzed by the Van Slyke method and the results show that they contain the basic amino-acids, arginine, histidine, lysine, and cystine.

2. The relatively high percentage of lysine in the proteins of the peanut indicates that this seed might be used to advantage in supplementing diets deficient in lysine.

BIBLIOGRAPHY.

1. Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, 1916–17, xxviii, 77.
2. Johns and Jones, *J. Biol. Chem.*, 1916–17, xxviii, 79.
3. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, xvii, 325.
4. Hopkins, F. G., *J. Chem. Soc.*, 1916, cix, 629.
5. *Weekly News Letter, U. S. Dept. Agric.*, 1916, iv, 6.

A NOTE ON THE SOLUBILITY OF CHOLESTEROL-DIGITONIDE.

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(Received for publication, March 30, 1917.)

In a review of the colorimetric and gravimetric methods for the determination of cholesterol,[1] it was shown that the colorimetric method of Autenrieth-Funk[2] gave results about 20 per cent higher than those obtained by the Fraser and Gardner[3] modification of the Windaus[4] digitonin method, while a colorimetric procedure proposed by Bloor[5] gave results even as much as 50 per cent higher than the latter. From the experimental evidence it was concluded, however, that the lower gravimetric values were probably more nearly correct. Bloor[6] has recently criticized this conclusion on the basis that the digitonin-cholesterol compound is sufficiently soluble in the ether used for washing to account for the low results.

Owing to the fact that pure cholesterol solutions give practically theoretical results when precipitated by digitonin and washed with ether and hot water, this criticism does not seem well founded. Nevertheless, solubility determinations of the compound in ether and in boiling water have been made and are here presented.

The compound was prepared by precipitating recrystallized cholesterol made from gall-stones, by digitonin, filtering, washing thoroughly with alcohol and ether, and drying. Some of the resulting compound was shaken with ether for 24 hours at room

[1] Mueller, J. H., *J. Biol. Chem.*, 1916, xxv, 549.

[2] Autenrieth, W., and Funk, A., *Münch. med. Woch.*, 1913, lx, 1243.

[3] Fraser, M. T., and Gardner, J. A., *Proc. Roy. Soc., Series B*, 1909, lxxxi, 230.

[4] Windaus, A., *Z. physiol. Chem.*, 1910, lxv, 110.

[5] Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 227.

[6] Bloor, W. R., and Knudson, A., *J. Biol. Chem.*, 1916, xxvii, 107.

temperature. After filtering, exactly 100 cc. were evaporated in several portions from a small weighed porcelain dish, the residue dried at 110°, and weighed. As an average of three determinations, 0.0007 gm. was found to have dissolved in 100 cc. of the ether. Similar determinations, using boiling water as solvent, showed a solubility of 0.0006 gm. in 100 cc.

Since approximately 100 cc. each of ether and hot water are used for washing in actual determinations of cholesterol, it is evident from these figures that an error of about 3 per cent might be accounted for in this way on 0.01 gm. of cholesterol (= 0.04 gm. compound), leaving the colorimetric results by Bloor's method still perhaps 45 per cent too high.

UREA AS A SOURCE OF BLOOD AMMONIA.

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(Received for publication, March 6, 1917.)

In the course of a recent study of urea excretion in rabbits it was sometimes found that within ½ to 2 hours after a dose of 5 gm. of urea by stomach tube the animals would have severe general convulsions, usually terminating in opisthotonus and death. In a series of 131 experiments such convulsions occurred in eight instances, while in the remaining animals no symptoms of intoxication were noted. Ammonia poisoning was suspected, since we had seen similar symptoms after the intraperitoneal injection of a urea solution which contained ammonia produced by the action of contaminating bacteria. This suspicion was confirmed when it was found that all rabbits with convulsions in which blood ammonia determinations were made showed a marked increase in ammonia. As much as 11 mg. per 100 cc. were found in one instance, although the urea solution used contained scarcely recognizable traces of ammonia. Attention has been called to the same phenomenon by Bang (1) who found a similar increase of blood ammonia in rabbits after 10 gm. of urea given by mouth, and showed that a blood concentration of about 4 mg. ammonia may be lethal. The same dose of urea given intravenously produced no symptoms, and he therefore concluded that the urea was changed into ammonia by the flora of the large bowel. However, his statement that this conclusion was also confirmed experimentally is not supplemented by any report of the experimental details. Cathcart (2) has recently brought forward evidence which would indicate the possible derivation of ammonia from urea given intravenously. Her figures show a slight increase of liver and muscle ammonia, but are far from convincing when we consider the sources of error of the tissue-ammonia method used.

We have therefore undertaken a brief investigation of the blood ammonia in rabbits following urea given (1) directly into the bowel,

and (2) intravenously, with and without exclusion of the bowel circulation. Serial determinations of the blood ammonia have been made by the method described by one of us (3), and the results are here given as graphs showing the amount of ammonia obtained from the blood at varying intervals following the urea injections.

I. Blood Ammonia Following Direct Injections of Urea into the Digestive Tract.

1. Weight of rabbit 1,200 gm. Ether anesthesia. Midline incision. 3 gm. of urea in 20 per cent solution injeeted directly into the large bowel and appendix. Irregular spasmodic respiration after 45 minutes. Death with respiratory failure at 53 minutes.

2. Weight of rabbit 1,200 gm. Same procedure. Jerky breathing after 30 minutes. Death with respiratory failure at 37 minutes.

3. Weight of rabbit 1,000 gm. Same procedure, with injection of 3.0 gm. of urea into bowel. No symptoms. Bled to death.

4. Weight of rabbit 1,300 gm. 5 gm. of urea (20 per cent) injected directly into the stomach after ligation of the pylorus. No symptoms.

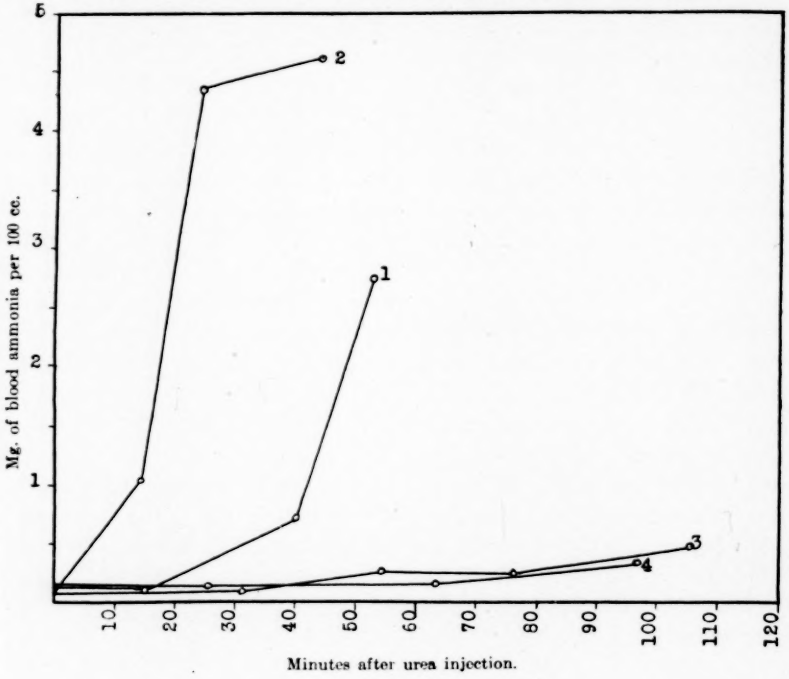

FIG. 1

Results.—Blood ammonia in rabbits shows a rapid rise following direct injection of large doses of urea into the large intestine. No significant rise occurs after urea injection into the stomach with the pylorus ligated.

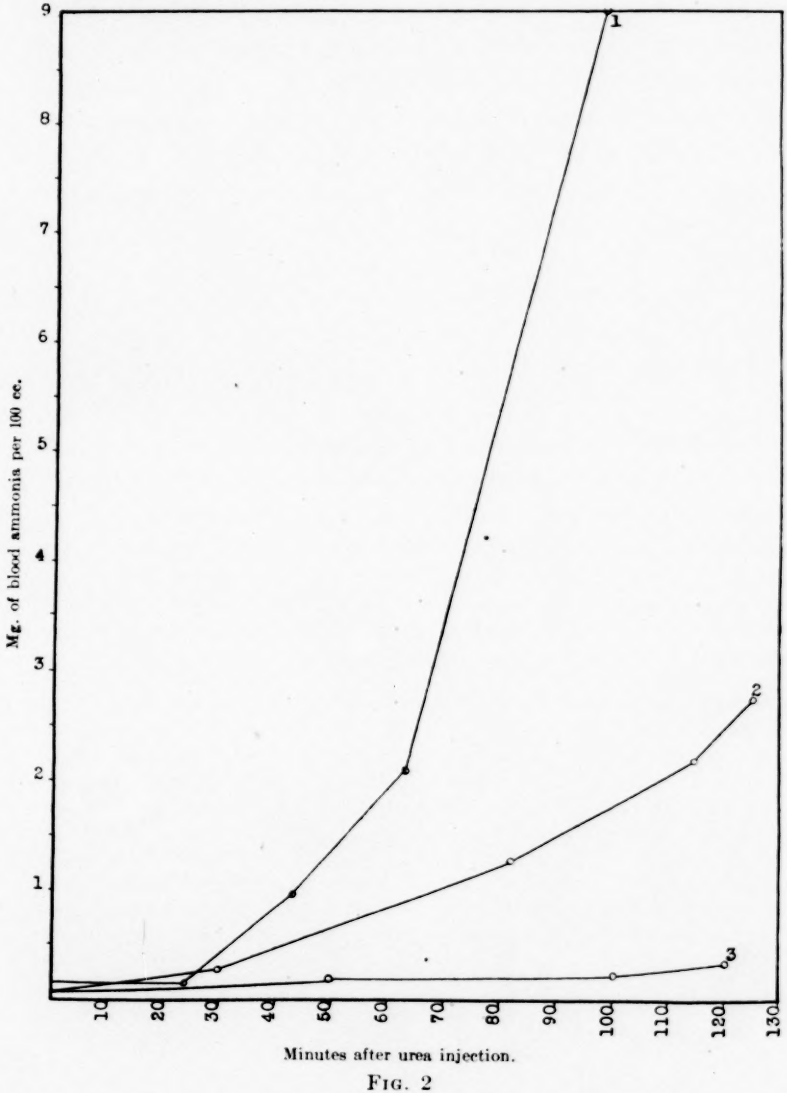

FIG. 2

II. Blood Ammonia Following Intravenous Injections of Urea.

1. Weight of rabbit 1,000 gm. Ether anesthesia. 15 gm. of urea in 25 per cent solution by cannula into the jugular vein during 13 minutes. Death with respiratory failure at 90 minutes.

2. Weight of rabbit 2,050 gm. Ether anesthesia. Celiac axis and superior and inferior mesenteric arteries clamped through lateral abdominal incision. 10 gm. of urea (20 per cent) injected intravenously during 7 minutes. Death from pericardial hemorrhage following removal of a blood sample from the heart.

3. Control. Weight of rabbit 2,250 gm. Vessels clamped as in No. 2. No urea given. Bled to death after 120 minutes.

Results.—The blood ammonia may show marked and rapid rise in rabbits following large doses of urea intravenously. A smaller and delayed rise may occur with the bowel circulation excluded.

DISCUSSION.

The striking increase of blood ammonia following the intravenous urea is, we believe, the clearest evidence that has been brought forward to suggest the possible derivation of ammonia from urea in the body.

The studies of Jacoby (4) and Lang (5) showed a slight urea-splitting activity of tissues *in vitro*, but are of little value, since bacterial action could not be excluded. Eppinger (6) reported that subcutaneous urea will prevent acid intoxication in rabbits when two or three times the lethal dose of hydrochloric acid is given by mouth, and concluded that the acid is neutralized by ammonia obtained from the urea. Pohl and Münzer (7) were unable to confirm Eppinger's experiments, but as noted by Eppinger (8) they gave the urea by mouth, a method which he also found inefficient. Their criticism that the extreme toxicity of ammonia for rabbits would speak against Eppinger's theory seems justified. The marked diuretic action of urea would appear to be a better explanation of its beneficent effect on acid intoxication. Observations on urinary ammonia following urea taken by mouth in man have yielded conflicting results. Janney (9) concluded that no ammonia is formed from the urea taken, although his protocols occasionally show a distinct increase in ammonia output following the urea dose. Lichtwitz (10) and Wolf (11) reported a considerable increase in ammonia output after taking urea, and concluded that the ammonia is formed from the urea. But here, too, the effects of urea diuresis cannot be disregarded. Wakeman and Dakin (12) and Jansen (13) in perfusion experiments found no evidence of a reversibility of the ammonia-urea reaction in the liver. Momose (14), using the oxygen-absorbing

capacity of the blood as a measure of its reaction, concluded that there is an increased alkalinity of the blood following urea given by mouth. The figures before and after urea, however, are apparently within the limits of error of the method.

We would explain the difference between our results with intravenous injection and those of Bang (1), who found no ammonia poisoning, by the fact that we have used larger doses of urea. Furthermore, as he made no determinations of blood ammonia in his intravenous experiments, a considerable sublethal rise may have occurred, a condition which we have found in experiments not here given in detail.

Some of our rabbits have died during or immediately following the urea injections, with general muscular twitching and fine irregular clonic spasms of the extremities, but without increase of blood ammonia. These are apparently due to urea poisoning, and are quite different from the ammonia convulsions, which have only occurred in animals surviving the direct urea intoxication.

It is clear that we have not determined the source of the ammonia. The attempt to exclude the bowel by clamping the arteries is not a satisfactory procedure, and is open to the criticism that the ammonia resulting from the autodigestion of the stomach and small bowel may find its way into the peritoneal cavity and be absorbed there. In fact we found in one such experiment an ammonia content of 26 mg. per 100 cc. in the fluid which accumulated in the peritoneal cavity. The same case showed perforation of the stomach. The delay in the ammonia rise when the bowel is excluded might suggest that the bowel is an important source of the ammonia, and it is of interest to recall that Herter (15) noted in certain animals a transudation of urea into the intestine following intravenous urea. But the rapidity of the rise in ammonia following simple intravenous urea would seem to us to indicate the possibility of some other mechanism. Further work is in progress toward the solution of the problem.

SUMMARY.

1. The blood ammonia of rabbits shows a marked increase following large doses of urea given by mouth, or directly into the bowel, or intravenously. Death from ammonia poisoning frequently follows such injections.

2. A less marked and later rise in blood ammonia may occur following intravenous urea in rabbits in which the bowel circulation is excluded.

3. The possibility that this ammonia may arise elsewhere than in the bowel is pointed out.

BIBLIOGRAPHY.

1. Bang, I., Untersuchungen über den Reststickstoff des Blutes. IV. Mitteilung, *Biochem. Z.*, 1915-16, lxxii, 139.
2. Cathcart, G. D., Nitrogen distribution in the tissues, *Biochem. J.*, 1916, x, 197.
3. Barnett, G. D., The micro-titration of ammonia, *J. Biol. Chem.*, 1917, xxix, 459.
4. Jacoby, M., Ueber die fermentative Eiweissspaltung und Ammoniakbildung in der Leber, *Z. physiol. Chem.*, 1900, xxx, 149.
5. Lang, S., Über Desamidierung im Tierkörper, *Beitr. chem. Physiol. u. Path.*, 1904, v, 321.
6. Eppinger, H., Beitrag zur Lehre von der Säurevergiftung. I. Mitteilung, *Wien. klin. Woch.*, 1906, xix, 111.
7. Pohl, J., and Münzer, E., Über Entgiftung von Mineralsäuren, *Zentr. Physiol.*, 1906-07, xx, 232.
8. Eppinger, H., and Tedesko, F., Zur Lehre von der Säurevergiftung. III. Mitteilung, *Biochem. Z.*, 1909, xvi, 207.
9. Janney, N., Die Ammoniakausscheidung im menschlichen Harne, *Z. physiol. Chem.*, 1911-12, lxxvi, 99.
10. Lichtwitz, L., Über chemische Gleichgewichte und Endzustände im Stoffwechsel, *Z. physiol. Chem.*, 1912, lxxvii, 402.
11. Wolf, C. G. L., Die Ausscheidungszeit von Stickstoff, *Biochem. Z.*, 1912, xl, 234.
12. Wakeman, A. J., and Dakin, H. D., Note upon relationship between urea and ammonium salts, *J. Biol. Chem.*, 1911, ix, 327.
13. Jansen, B. C. P., *Arch. Néerland*, 1915, ii, 594.
14. Momose, G., The effect of ingestion of urea, etc., on the reaction of the blood, *Biochem. J.*, 1915, ix, 485.
15. Herter, C. A., On urea in some of its relations, *Rep. Johns Hopkins Hosp.*, 1900, ix, 69.

THE INFLUENCE OF THE PROTEIN INTAKE ON THE EXCRETION OF CREATINE IN MAN.

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(Received for publication, March 29, 1917.)

In a previous paper[1] we have shown that in man the excretion of creatine is largely confined to the day, only a small proportion of the total amount of this substance found in the 24 hour urine being eliminated during the night. It was also noted that during the day the output of creatine rose markedly after each meal, thus showing the unmistakable influence of the ingestion of creatine-free food on the excretion of this body. In the present paper we wish to present the results of a series of experiments which we think add further stability to the evidence concerning the influence of protein ingestion on creatine excretion.

In the course of a series of creatine determinations made on the urine of a number of patients suffering from hyperthyroid disease, it was noted incidentally that those patients who were possessed of the insatiable appetite so often seen in the victims of this malady had considerable quantities of creatine in the urine, while persons suffering from an equally severe form of the same disorder, but possessed of little or nearly normal appetite, excreted much smaller quantities of this body. On the working hypothesis that the urinary creatine is dependent on the intake of protein in the food, we have carried out a series of feeding experiments in which patients were fed for varying periods first on the highest protein diet (creatine-free) that they could be induced to take, and then on one containing the minimum amount of protein. The subjects used in the experiments recorded below were kept in bed during the entire period of observation.

[1] Denis, W., *J. Biol. Chem.*, 1917, xxix, 447.

Subject.	Experiment.	Sex.	Age.	Weight.
			yrs.	*kg.*
I	I	F.	33	55.2
II	II	M.	35	58.0
III	III	F.	21	59.0
IV	IV	F.	46	52.3
V	V	F.	23	54.5

All were suffering from hyperthyroid disease of moderate severity and were receiving no drugs during the experimental period.

Observations of body temperature were made at 7 a.m. and 7 p.m. daily, but as no temperatures above normal were recorded these figures have been omitted from the table.

An attempt was made to use practically the same articles of food in all five cases, but personal idiosyncrasies made it necessary to vary to a certain extent the quantity of food given.

During the periods of high protein feeding the following foods were used: bread, a wheat cereal, butter, 40 per cent cream, eggs, cheese, gelatin, sugar, oranges, and apples. During the low protein periods the food consisted of bread, rice, tapioca, butter, 40 per cent cream, potatoes, olive oil, fat bacon, sugar, arrowroot starch, lactose, lettuce, oranges, apples, and bananas.

Creatine and creatinine determinations were made by Folin's micro method,[2] specially purified picric acid being used. The nitrogen determinations were made by the colorimetric method of Folin and Denis.[3]

The figures given in Table indicate gm. of creatine, creatinine, and total nitrogen found in a 24 hour quantity of urine.

As will be seen by an inspection of the figures tabulated below it is apparent that the quantity of protein taken in the food exerts an unmistakable effect on the excretion of creatine in man, a finding in accord with the observations made by McCollum and Steenbock[4] on the pig. Mendel and Rose[5] have shown that the administration of carbohydrate to starving rabbits causes a marked

[2] Folin, O., *J. Biol. Chem.*, 1914, xvii, 472.

[3] Folin and Denis, *J. Biol. Chem.*, 1916, xxvi, 473.

[4] McCollum, E. V., and Steenbock, H., *J. Biol. Chem.*, 1912–13, xiii, 209.

[5] Mendel, L. B., and Rose, W. C., *J. Biol. Chem.*, 1911–12, x, 213, 255.

diminution in the excretion of creatine. Arguing from the results obtained by these investigators, it might be claimed that the high creatine figures found during the periods of forced protein feeding are due to a lack of carbohydrate in the diet. While the fact that during the periods of low protein feeding we not

TABLE I.

Day.	Experiment I.			Experiment II.			Experiment III		
	Total nitrogen.	Prepared creatinine.	Creatine.	Total nitrogen.	Prepared creatinine.	Creatine.	Total nitrogen.	Prepared creatinine.	Creatine.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	9.1	0.66	0.26	10.2	1.22	0.46	11.9	0.84	0.64
2	11.2	0.74	0.48	8.7	1.25	0.22	12.2	0.78	0.65
3	9.2	0.69	0.54	6.6	1.24	0.19	11.1	0.82	0.53
4	12.7	0.71	0.61	13.2	1.20	0.34	10.4	0.81	0.48
5	7.0	0.68	0.56	20.7	1.33	0.75	8.8	0.80	0.48
6	6.5	0.69	0.29	21.8	1.29	0.79	8.5	0.83	0.31
7	5.6	0.68	0.11				6.6	0.78	0.27
8	4.7	0.68	0.08				6.3	0.78	0.32

Day.	Experiment IV.			Experiment V.		
	Total nitrogen.	Prepared creatinine.	Creatine.	Total nitrogen.	Prepared creatinine.	Creatine.
	gm.	gm.	gm.	gm.	gm.	gm.
1		0.62	0.03	14.44	0.68	0.39
2	8.5	0.62	0.03	11.07	0.71	0.29
3	12.2	0.58	0.16	11.0	0.70	0.32
4	13.7	0.60	0.24	10.8	0.70	0.32
5	11.4	0.62	0.25	7.0	0.68	0.16
6	12.1	0.75	0.25	5.5	0.74	0.00
7	8.08	0.74	0.19			
8	4.16	0.60	0.00			

only decreased the intake of protein but also increased the consumption of carbohydrates cannot be denied, we feel the above objection to be invalid, as, on looking over the diet lists, it is apparent that at least 300 gm. of carbohydrate were consumed daily by all the subjects during the high protein periods, an amount certainly sufficient to preclude the idea of "carbohydrate starvation."

The conditions under which creatine excretion has been noted in man, *viz.*, in starvation, in childhood, in pregnancy, in fevers, and in hyperthyroid disease, are all conditions accompanied by a high level of protein metabolism. The observations of Krause,[6] that small quantities of creatine are frequently found in the urine of women, may also possibly be attributed to the relatively high protein intake of women when considered from the standpoint of their muscular development.

It was pointed out some 10 years ago by Folin that the results obtained in feeding experiments with creatine depend entirely on the previous diet of the subject. If the food had been low in protein, a marked retention of creatine was observed; if high, most of the ingested creatine was found in the urine.

As a working hypothesis it may be suggested, therefore, that on the ingestion of protein some fraction of this is transformed into creatine, transported to the muscles, and there absorbed.[7] If so much creatine is manufactured that the muscles become supersaturated, creatine is excreted by way of the kidney. What would constitute supersaturation would depend not only on the total mass of muscle, but on the capacity for creatine absorption possessed by these muscles. Thus it will be noted in the experimental results presented above that the first day of high protein feeding seldom brings about much of an increase in the creatine excretion; it is only after a day or two of this diet that a marked increase in excretion is observed.

It must also be borne in mind that the muscles of different individuals on the same diet may and probably do have different saturation points. It has been pointed out by Folin and Denis[8] that if, as they suggest, the creatine found in the urine of children is due to an excessively high level of protein consumption, then it should be possible to reproduce in adults by forced feeding with protein the condition with reference to creatine found in children. It is exceedingly improbable, however, that any creatine excretion would result were the diet given to these hyperthyroid patients eaten by a normal woman. It would therefore seem possible that in this condition and in some other pathological condi-

[6] Krause, R. A., *Quart. J. Exp. Physiol.*, 1911, iv, 293.
[7] Folin and Denis, *J. Biol. Chem.*, 1914, xvii, 493.
[8] Folin and Denis, *J. Biol. Chem.*, 1912, xi, 253.

tions, as well as in childhood, the saturation point of the muscle for creatine is low, a hypothesis supported by the low values for muscle creatine found in children and in persons suffering from exophthalmic goiter.[9]

SUMMARY.

Results are presented on five cases of Graves' disease in which it is shown that the amount of creatine excreted by these individuals is dependent on the intake of protein, being increased by high protein feeding and decreased or even eliminated by low protein feeding.

[9] Denis, *J. Biol. Chem.*, 1916, xxvi, 379.

INHIBITION OF DIGESTION OF PROTEINS BY ADSORBED TIN.

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(From the Department of Chemistry, Princeton University, New Jersey.)

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INTRODUCTION.

It is known that tin is present in foods which have been packed in tin cans, and, since it is taken into the body, the question of the toxicity of the tin is important. Determinations of the toxicity of soluble tin salts have been made by a number of investigators.[1] We have shown in a previous paper that, in some of these foods, the tin removed from the lining of the can remains in true solution; that is, in a dialyzable form.[2] In the case of such foods, determinations of physiological action, as usually made, would apply. Many fruits and vegetables, however, while containing little acid, still dissolve large quantities of tin and in these the greater percentage of the tin has been shown to be present in an insoluble form, neither dialyzable nor filterable. This was especially true in foods whose protein content was high. We found, too, that this tin was very firmly bound, and, judging from the stability of this tin-protein complex, we suggested the possibility that it might pass through the body without being broken up by digestion. In order to secure evidence on these points, artificial gastric and tryptic digestions of proteins have been carried out in the presence and absence of adsorbed tin.

EXPERIMENTAL.

Several methods were available for the study of proteolytic action, the applicability of each depending on the nature of the in-

[1] White, T. P., *Arch. exp. Path. u. Pharm.*, 1880–81, xiii, 53. Ungar, E., and Bodländer, G., *Z. Hyg.*, 1887, ii, 241. Salant, W., Rieger, J. B., and Treuthardt, E. L. P., *J. Biol. Chem.*, 1914, xvii, 265.

[2] Goss, B. C., *J. Ind. and Eng. Chem.*, 1917, ix, 144.

vestigation. Since we were only interested in comparing the rate and extent of proteolysis of proteins containing adsorbed tin with that of the normal protein, a method which gave relative results and could be rapidly carried out was desirable. The biuret reaction, as described by Sherman and Neun in a recent review of methods,[3] fulfilled these conditions, the gradual change in color from violet to rose-red indicating the formation of peptones. The methods which depend on a change in hydrogen ion concentration, such as the formol titration, were not applicable here because of the fact that adsorption phenomena occurred in the case of the tin-protein complex, producing a change in acidity when no enzyme was present. For example, a series of albumins, containing varying amounts of adsorbed tin, was placed in contact with a 0.5 per cent Na_2CO_3 solution and it was found that as the percentage of tin increased, the decrease in acidity became more marked, although no tin went into solution.

TABLE I.

Decrease in Acidity of Solutions in Contact with Tin-Protein Adsorption Complex.

8 gm. of albumin in 150 cc. of 0.5 per cent Na_2CO_3. 25 cc. of this solution were found equivalent to the following volumes of 0.1 N HCl.

Time, *hrs.*	0	½	1	Decrease in HCl.
Mixture	0.1 N HCl required.			
	cc.	cc.	cc.	cc.
Albumin without tin	9.55	8.75	8.65	0.9
" with 1.16 per cent tin	9.55	8.15	7.4	2.15
" " 32.8 " " "	9.6	6.65	4.3	5.3

This phenomenon will receive further attention.

The biuret reaction, taken together with the visible differences in the amounts of coagulated proteins left undissolved at any period of time, and supplemented by tin determinations on the digestion liquor and residue, was found to give interesting results from the point of view indicated above.

The proteins studied were egg albumin, edestin, casein, and the 10 per cent NaCl extract of globulins (and albumins) from pea-

[3] Sherman, H. C., and Neun, D. E., *J. Am. Chem. Soc.*, 1916, xxxviii, 2199.

beans, each in the solid form. The egg albumin was prepared by boiling eggs for 10 minutes, removing the albumin, and pressing it through a 20 mesh gauze, thus obtaining samples of uniform surface and water content. 7 gm. samples of this albumin were then weighed out and placed in a series of flasks, 100 cc. of a solution of stannic ammonium chloride in 1 per cent HCl being added to each, the concentrations ranging from 10 to 0.01 per cent. After remaining in contact with the albumin for 12 hours, during which time the mixture was frequently stirred, a 10 cc. portion of the solution was withdrawn from each concentration and analyzed for tin; then the tin solutions were drained off and the albumin

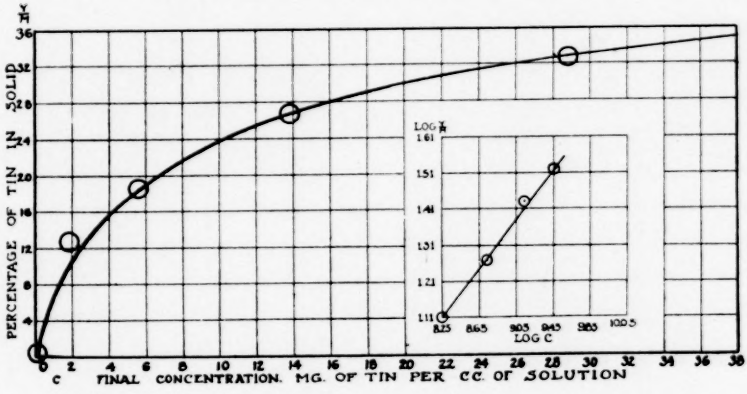

FIG. 1. Adsorption of tin by coagulated egg albumin.

was washed repeatedly with distilled water, finally being left over night under distilled water, this water giving no test for tin. A part of the solid was dried at 110°C. and the percentage of tin determined, after destruction of the organic matter, by precipitating as sulfide from acid solution, the sulfide being roasted and weighed as SnO_2. From these results the adsorption curve was plotted.

The heat-coagulated globulins from beans, crystallized edestin, and the acid-precipitated casein from milk were all treated in a similar way and were all found to adsorb tin in varying degrees.

Equal volumes (100 cc.) of a solution of 0.5 per cent pepsin in 0.05 N HCl were added to the protein and tin-protein samples

and the flasks placed in a thermostat at 38°C. At intervals the filtrate was tested by the biuret reaction and the appearance of the solid noted. After the experiment was finished, the percentage of tin in the solid residue was determined and the liquor tested for filterable and dialyzable tin.

In the same way another series was treated with a solution of trypsin, 0.5 per cent, in 0.1 N Na_2CO_3.

Some typical results follow.

TABLE II.

Peptic Digestion of Egg Albumin as Indicated by the Biuret Reaction.

Time.	Percentage of tin in coagulated albumin.						
	0 Boiled.	0	1.16	12.8	18.5	26.8	32.8
hrs.							
1	Violet.	Red.	Red-violet.	Violet.	Violet.	Violet.	Violet.
2	"	"	Violet-red.	Red-violet.	"	"	"
6	"	"	Red.	Violet-red.	Red-violet.	Red-violet.	"
18	"	"	"	Red.	Violet-red.	Violet-red.	Red-violet.
					Residue, 50.5 per cent tin. Dialyzable tin, none. Filterable tin, traces.		Residue, 48.2 per cent tin. Dialyzable tin, none. Filterable tin, traces.

The albumin containing no tin was almost completely dissolved after 2 hours, while the albumin with 26 and 32 per cent tin showed little change in volume at the end of 18 hours. The volume of the residue after 18 hours was proportional to the percentage of tin in the albumin at the beginning of the experiment.

TABLE III.

Coagulated Egg Albumin.
Tryptic Digestion as Indicated by the Biuret Reaction.

Time.	Percentage of tin in coagulated albumin.						
	0 Boiled.	0	1.16	12.88	18.53	26.85	32.8
hrs.							
1	Violet.	Red-violet.	Reddish violet.	Violet.	Violet.	Violet.	Violet.
2	"	Violet-red.	Red-violet.	Reddish violet.	"	"	"
4	"	"	"	Red-violet.	Red-violet.	Red-violet.	"
6	"	"	Violet-red.	"	"	"	Reddish violet.
18	"	Red.	"	Violet-red.	"	"	Red-violet. Residue 62.7 per cent tin. Dialysate, no tin. Filtrate, no tin.

In tin 0 per cent there was almost complete solution after 4 hours, while in tin 32 per cent there only was a slight apparent volume change after 18 hours.

TABLE IV.

Casein, Peptic Digestion.

Time.	Percentage of tin in casein.			
	0	1.02	8.5	21.4
hrs.				
3	Violet-red.	Violet.	Violet.	Violet.
5	Red.	Red-violet.	"	"
9	"	Violet-red.	Violet-red.	Red-violet.
18	"	Red.	Red.	Violet-red.

TABLE V.

Edestin, Tryptic Digestion.

Time.	Tin 0, boiled.	Tin 0.	Tin-protein from 5 per cent solution.
hrs.			
1	Violet.	Violet-red.	Violet.
2	"	Red.	"
3	"	"	Red-violet.
18	"	"	Violet-red.

After 18 hours the normal edestin was almost completely dissolved while there was no apparent change in the tin-edestin.

The results given by the biuret reaction in the case of the globulins were not decisive, but, while the normal globulin was almost completely dissolved after 6 hours, the tin globulin showed little or no change after 18 hours.

TABLE VI.

Peptic Digestion, Egg Albumin Containing 37 per Cent Tin.

Time.	Tin 0, boiled.	Tin 0.	Tin 37 per cent.
hrs.			
2	Violet.	Red-violet.	Violet.
8	"	Red.	"
24	"	"	"
48	"	"	Red-violet.
100	"	"	Red.

After 100 hours there was almost no change in the volume of the solid albumin in the digestion mixture in the flasks containing the tin-protein complex. A duplicate which was examined at the end of 48 hours was found to have no dialyzable tin, 3 mg. of filterable tin, and 1,175 mg. of tin in the washed solid residue.

CONCLUSIONS.

We have found that tin is readily adsorbed from solutions by coagulated proteins in amounts varying with the concentration according to the adsorption law of Freundlich; that this adsorption takes place rapidly at first and then continues to increase slowly for several days, presumably because of the time required for diffusion into the solid. The adsorption complex is extremely stable and does not lose tin to a dilute acid or alkaline aqueous phase containing no tin although the percentage of tin in the solid

may be as high as 50 per cent. The presence of this tin, even in small amounts, interfered markedly with the digestion of the protein by either peptic or tryptic digestion, as was indicated by the change in color of the biuret reaction and by the visible retardation of the solution of coagulated proteins. At the end of a 6 hour digestion, under exactly the same conditions, it was possible to arrange a series of albumins in the order of the increasing percentages of tin, by observing the extent to which solution had taken place. It was observed, however, that when the time was increased a certain amount of solution or digestion did occur even when the protein contained large amounts of tin, and that in the case of the proteins of low tin content, comparable with the condition present in a can of food, only a small residue was left undissolved. This residue contained practically all the tin. Also, if a sample of albumin was left but a short time in contact with a strong tin solution before being subjected to digestion, the residue presented a more or less shell-like structure. It appears from these observations that it is only a part of the protein, directly joined to the tin, whose digestion is hindered. The effect of this retardation, on the food value of a protein containing tin, can only be a matter of conjecture but it is probably small since the total tin in foods rarely exceeds 0.03 per cent.

The other aspect of the problem is more important; namely, that of the possible toxicity of this tin-protein complex. Food rarely remains in contact with the digestive ferments in the body for a longer period than 4 to 6 hours. We have found that even after 48 hours, in the case of the artificial peptic digestions of the tin-albumin containing 32 per cent tin, there was no tin in a dialyzable form, and only a very small amount in a filterable form, this probably being present as a colloidal suspension of particles of the tin-protein complex, split away from the solid by solution of the connecting albumin. In the tryptic digestions no dialyzable tin was found in the liquor and only traces of filterable tin. Moreover, during digestion the percentage of tin in the solid rapidly increased, indicating that the protein which was not directly bound to the tin was being dissolved away. A sample of albumin which originally contained 18 per cent of tin was found after 24 hours' digestion to contain 50 per cent; one with 32 per cent increased to 62 per cent. The activity of the

enzyme was shown to be unaffected by the tin in the proteins by removing some of the liquor from the undigested tin-protein after 100 hours and finding that fibrin was completely and quickly dissolved by the liquor.

We have found that the insoluble complex, formed when coagulated proteins are brought into contact with solutions containing tin, was not broken up to any extent by our artificial gastric and tryptic digestions. The two enzymes, peptin in the stomach and trypsin in the duodenum, are the ones which bring about proteolytic action in the body. Erepsin does not attack proteins themselves, but hydrolyzes the peptones and polypeptides which are formed by the partial splitting of the proteins. While it is impossible to predict what the action of bacteria in the stomach and intestine would be, it is to be questioned whether the tin-protein complex is broken up in the actual digestive processes in the body and whether the tin which is combined in this way can have any toxic action.

SEDOHEPTOSE, A NEW SUGAR FROM SEDUM SPECTABILE. I.*

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Sedum spectabile belongs to the natural order of *Crassulacea*, the members of which are widely distributed throughout the warm and temperate zones. It is a cultivated herbaceous perennial plant, very hardy, is extremely easily propagated by cuttings or division of the roots, and is commonly used in many countries as an ornamental plant. The order has been but little studied chemically.

Indications of the Presence of a Sugar Having Peculiar Properties.

It was first noticed that an aqueous extract of the crushed fresh leaves and stalks of the plant, prepared during the growing season, from May to September, contained considerable amounts of a free reducing sugar, which was not fermentable with yeast and gave a strong test with orcin and hydrochloric acid, indicating the presence of a pentose or heptose sugar.

It was next sought to learn whether the sugar is an aldose or a ketose. To determine whether one or both of these types are present in a solution, two methods may be applied. In general, ketoses are destroyed by boiling with hydrochloric acid, leaving any aldoses which may be present affected only slightly or not at all. By determining the copper-reducing power of the sugar solution before and after such treatment, a clue to the amounts of aldehydic and ketonic sugars may be obtained.

A second method, which may serve as a control to the first, consists in allowing bromine to act on the sugar solution. This

*Our thanks are expressed to Mr. T. Yoshikawa for valuable assistance rendered during part of this work.

reagent oxidizes aldoses to non-reducing monobasic acids while ketoses remain unaffected.

The plant extract was therefore subjected to both these treatments. In each case a loss in power to reduce Fehling's solution was observed and the amount of Cu_2O formed from a given volume of solution after treatment with either acid or bromine was about 20 per cent of the amount from the same quantity of the original solution.

These results are unintelligible on the assumption that a mixture of aldoses and ketoses of the usual type is present in the plant extract, because the result of the action of hydrochloric acid would indicate that the mixture consisted of 20 per cent aldose and 80 per cent ketose, while the change caused by bromine would lead one to the opposite conclusion.

In both instances similar changes in the optical rotation of the two solutions were observed. From an original reading of about 0°, both treatments caused the solutions to become strongly levorotatory. These puzzling relations made it seem probable that a sugar was present in the *Sedum* plant which exhibited abnormal behavior and it was decided to submit the substance to a thorough investigation. Consequently, a large amount of the plant was treated according to the following method.

Preparation of a Sugar Sirup from the Plant.

This preparation may be best illustrated by the following experiment on a small scale, in which 1,650 gm. of the fresh leaves and stalks of *Sedum spectabile* were used. The material was passed through a chopping machine, an equal volume of water added, and the liquid allowed to drain over night through cloth. Most of the remaining liquid was extracted from the residue by means of a powerful press and the operation was repeated once after moistening the material with about 500 cc. of water. The combined extracts were boiled with a little decolorizing carbon, filtered on folded filters, and then concentrated under reduced pressure to a thin sirup. This was diluted with 95 per cent alcohol until a permanent precipitate was produced, and then poured into four or five times its volume of alcohol of the same strength. The voluminous light colored material which separated was fil-

tered off with suction and washed by thoroughly mixing with alcohol and again filtering. The combined filtrates were evaporated under diminished pressure to a thick sirup. Portions of about 250 gm. of this material were diluted with ten parts of water and in the solution 5 to 6 gm. of tannic acid were dissolved, and basic lead acetate solution was then added as long as a precipitate was produced.[1] This was removed by filtration with suction and the filtrate, after removal of the lead with hydrogen sulfide, was concentrated under diminished pressure to a thick sirup which was used directly in some of the following experiments and is referred to as "crude sirup." It may be purified to a certain extent in the following manner. 70 gm. were dissolved in 700 cc. of water, the solution was cooled by cracked ice, and then made slightly alkaline by addition of a warm solution of barium hydroxide. The solution was then neutralized with carbon dioxide and concentrated to a thin sirup (about 140 cc.). As much 95 per cent alcohol was added as was possible without producing a permanent precipitate and the solution was poured into 2 liters of absolute alcohol which was being vigorously stirred. The separated white material, which settled readily, was filtered off with suction and washed with alcohol. The filtrate on evaporation under diminished pressure yielded a clear light yellow sirup which will be referred to as "purified sirup."

In preparations on a large scale, much greater quantities than the above mentioned amount of the plant were worked up and several kilos of "crude sirup" prepared.[2] Calculated as glucose from the reducing power of the extracts, there was about 1.5 per cent free sugar present in the fresh plant.

Action of Hydrochloric Acid and of Bromine on the Sirups.

A solution containing about 10 per cent of the purified sirup showed only a slight dextrorotation, but when it was slightly acidified with hydrochloric acid and allowed to stand for several

[1] Schorger, A. W., and Smith, D. F., *J. Ind. and Eng. Chem.*, 1916, viii, 495.

[2] Acknowledgments are made to Mr. Xavier Schmitt, Horticultural Hall, Fairmount Park, Philadelphia, to Thomas Meehan and Sons, Inc., Germantown, Pa., and to Wm. H. Moon and Company, Morrisville, Pa., for supplying large quantities of *Sedum spectabile*.

hours or warmed for a short time on the steam bath, its rotation was reversed and greatly increased in magnitude. At the same time its reducing power towards Fehling's solution decreased regularly with the increase in levorotation. These relations in a 10 per cent solution may be illustrated by the following table.

HCl content.	Time of heating.	α in 2 dm. tube.	Cu_2O from 1.66 cc.
per cent	*min.*		*gm.*
0	0	+ 0.5°	0.2976
1	7	− 6.1°	0.2003
1	14	−12.5°	0.1241
1	60	−19.2°	0.0603
1	90	−19.8°	0.0630

It is apparent from the changes in properties of the sugar solution that some reaction has occurred. This is probably not the destruction of some ketose which might be present, because sugars of this type are not much affected by such mild treatment, and, besides, their destruction by hydrochloric acid is always indicated by coloring, whereas solutions of the sugar from the *Sedum* plant remained perfectly clear and did not darken during the process.

The possibility that the change was the result of hydrolysis of a complex carbohydrate must also be excluded because, obviously, such a reaction would be accompanied by an increase in reducing power instead of a decrease as observed.

The reaction which occurs apparently reaches a definite endpoint after 1 hour, since prolonged heating produces no further change and the ratio of the reducing power of the material in the final solution to that in the original remains about as 20: 100.

If a solution of the sugar thus transformed is treated with an excess of bromine, no further changes in reducing power or rotation are produced. The action of this reagent was then tested on a solution of the untransformed sugar sirup.

Bromine causes a solution of the sirup to undergo a change that appears to be substantially the same as that produced by dilute hydrochloric acid. Such a solution, having an initial reducing power of 0.1360 gm. of Cu_2O per 1.66 cc. and an initial rotation of +0.4° in a 2 dm. tube, was allowed to stand with an excess of

bromine. After 3 days the excess of the reagent was removed with a stream of air, and the solution then showed a rotation in a 2 dm. tube of $-10°$ and a reducing power of 0.0416 gm. Cu_2O per 1.66 cc. In this case, as well as in that of the transformed sugar solution, much of the bromine had been used up and appeared partly as free hydrobromic acid in the solution. At the same time, no organic acids were produced, because after removal of the halogen with silver carbonate and the slight excess of silver with hydrogen sulfide the solution proved to be nearly neutral and no evidence of the presence of a lactone could be obtained. Evidently the action of bromine is one of substitution, probably in some organic substance present in the solution other than the sugar, and liberation of hydrogen bromide, which then acts upon the unchanged sugar present in the same manner as hydrochloric acid.

It was concluded from these experiments that there is present free in the *Sedum* plant a reducing sugar which undergoes in dilute hydrochloric acid solution a change by which its optical rotation is completely altered and its reducing power largely decreased. It was thus apparent that the sugar exhibits properties which have not hitherto been observed in this group, and it was next sought to obtain crystalline derivatives of it, especially those with the phenylhydrazines.

Behavior of the Sugar towards the Phenylhydrazines.

Phenylosazone.—All attempts to prepare a hydrazone under varied conditions resulted in the formation of the osazone. This derivative was prepared with ease by the usual method from about a 2 per cent solution of the sirup. It also formed in solutions at room temperature when they were allowed to stand for a few hours with phenylhydrazine acetate. The yield was better than that usually obtained from other sugars, and even from sugar solutions that had been heated with dilute hydrochloric acid the same osazone was obtained and the amount about corresponded to the final reducing power of the solution; that is, it was about 20 per cent of the amount that could be obtained from the solution before transformation by acid. The compound was recrystallized by dissolving it in hot methyl alcohol, filtering, and concen-

trating the solution to a small volume. The substance melted with decomposition at 197° (uncorrected).

The analysis corresponds to the phenylosazone of a heptose.

0.1407 gm. substance gave 0.3031 gm. CO_2 and 0.0797 gm. H_2O.
0.1366 " " " 17.95 cc. N_2 at 28° and 764 mm.
0.1269 " " " 17.0 " " " 29° " 765 "

	Calculated for $C_{19}H_{24}O_5N_4$:	Found:
C	58.76	58.75
H	6.19	6.29
N	14.43	14.66 14.65

p-Bromophenylosazone.—All efforts to prepare a *p*-bromophenylhydrazone in aqueous alcoholic and glacial acetic acid solution yielded the corresponding osazone.

100 cc. of a 2 per cent aqueous solution of the sugar sirup were heated for 3 hours on the steam bath after addition of 5 gm. of *p*-bromophenylhydrazine in 4 cc. of acetic acid. The osazone crystallized from the hot solution in bright yellow needles and was recrystallized from methyl alcohol in the same manner as was the unsubstituted osazone. It melted with decomposition at 227–228° (uncorrected). The analysis corresponds to the bromophenylosazone of a heptose.

0.2577 gm. substance gave 0.1766 gm. AgBr.
0.2466 " " " 0.1692 " "
0.1610 " " " 15.6 cc. N_2 at 30° and 763 mm.

	Calculated for $C_{19}H_{22}N_4Br_2O_5$:	Found:
Br	29.30	29.16 29.18
N	10.26	10.50

No crystalline methyl-, benzyl-, or *p*-nitro-phenyl hydrazone could be prepared under any conditions tried.

The Osone of the Sugar of the Sedum Plant.—The ease with which the new sugar combined with phenylhydrazine indicated the possibility of its being of the nature of an osone rather than of a true sugar. In order to compare this derivative with the sirup obtained from the plant extract the phenylosazone was decomposed according to Fischer's method for preparing glucose

osone.[3] The only changes which were introduced in his directions consisted in omitting to warm the solution of the osazone in concentrated hydrochloric acid, and, in precipitating the lead compound of the osone, a small amount of lead acetate solution was added before precipitation with barium hydroxide. The product obtained from the lead compound was a yellow sirup which did not crystallize. It reduced Fehling's solution strongly and rotated slightly to the left, but did not exhibit changes in hydrochloric acid similar to those described in the case of the reducing substance from which the osazone was derived. On treatment with phenylhydrazine acetate it yielded the original osazone.

Compound of the Osone with o-Phenylenediamine. This derivative was prepared by adding a hot aqueous solution of 2 gm. of *o*-phenylenediamine to about 4 gm. of osone sirup in 60 cc. of water. The slightly colored solution was warmed for 45 minutes on the steam bath and deposited long white needles on cooling. The yield was about 2 gm. The substance was recrystallized from hot water and dried over night in a desiccator. It melted with decomposition at 163–165° (uncorrected) after it had begun to shrink at about 160°. The compound appears to contain one-half molecule of water of crystallization.

0.1519 gm. air-dried substance, when heated to constant weight at 100° *in vacuo* over P_2O_5, gave 0.0042 gm. H_2O.

0.1340 gm. air-dried substance gave 0.0033 gm. H_2O.

	Calculated for $C_{18}H_{18}N_2O_5 + \frac{1}{2} H_2O$:	Found:
H_2O	3.12	2.75 2.46

0.1477 gm. dry substance gave 0.3023 gm. CO_2 and 0.0780 gm. H_2O.
0.1307 " " " " 0.2680 " " " 0.0673 " "
0.1382 " " " " 12.3 cc. N_2 at 24° and 765 mm.

	Calculated for $C_{18}H_{18}N_2O_5$:	Found:	
C	55.71	55.81	55.87
H	5.70	5.86	5.72
N	10.00	10.00	

The analysis thus corresponds to the phenylenediamine derivative of the osone of a heptose. It was not possible to obtain

[3] Fischer, E., *Ber. chem. Ges.*, 1889, xxii, 87.

this derivative from the original sugar sirup and therefore it was concluded that the reducing substance could not be a compound of the osone type.

Since the analyses of the three crystalline derivatives that have been described show conclusively that the sugar of *Sedum spectabile* contains seven carbon atoms, it will be named, with reference to its origin, *sedoheptose*.

Reduction of the Sugar Sirup with Sodium Amalgam.

In order to obtain less complicated crystalline derivatives of the new sugar and also with the object of determining if possible whether it is of aldehydic or ketonic nature, it was next sought to prepare the corresponding heptitol or heptitols by reduction with sodium amalgam. Since aldoses give on reduction only one alcohol while ketoses yield two isomeric ones, the two types of sugars may be distinguished by their behavior on this treatment.

From the sugar sirup two seven carbon alcohols were obtained and the inference would therefore be that it contained a ketose. However, as the material used for reduction was a sirup of uncertain purity there still remained the possibility that it contained two sugars. On the other hand, only one phenylosazone could be obtained from the sirup, and therefore if more than one sugar is present the mixture would seem to be limited to two epimeric aldoheptoses and the corresponding ketose. This point will be discussed later on, when it will be shown that the reducing substance is almost certainly a single ketose sugar.

α-Sedoheptitol.—28 gm. of purified sirup were dissolved in 150 cc. of water, and the solution was cooled by surrounding it with ice and was reduced with sodium amalgam. A small amount of litmus was added as an indicator and the solution was neutralized from time to time with sulfuric acid. The reduction, which was always carried out on the alkaline side, required several days action of the amalgam for completion. It was found convenient to make the reduction in an open vessel of heavy glass, keeping the solution and amalgam agitated with a mechanical stirrer. When the liquid showed no reduction of Fehling's solution, or only a trace, it was made slightly acid to Congo paper, separated from the mercury, and concentrated under reduced pres-

sure until a large part of the salts had separated. It was then mixed with a large volume of hot 95 per cent alcohol, a little decolorizing carbon was added, and the separated salts were removed by filtration with suction. The clear solution yielded on evaporation a nearly colorless sirup which crystallized partially after several weeks standing. If crystals for seeding are available, this time can be shortened to a few days. The crystals were stirred with a little 90 per cent alcohol, filtered off, and washed with a little of the same solvent. The yield was 7.5 gm. The substance was recrystallized by dissolving in a small amount (one to two parts) of hot water, filtering, and adding several volumes of hot absolute alcohol. It melted sharply without decomposition at 151–152° (uncorrected). It was again recrystallized in the same manner as described and showed no change in melting point. The analysis agrees with the composition of a heptitol.

0.1340 gm. substance gave 0.1941 gm. CO_2 and 0.0900 gm. H_2O.

	Calculated for $C_7H_{16}O_7$:	Found:
C	39.63	39.56
H	7.54	7.46

0.4857 gm. substance in 5 cc. H_2O in a 1 dm. tube showed a dextrorotation of +0.21°; hence $[\alpha]_D^{20} = +2.25°$.

0.4000 gm. substance in 15 cc. aqueous solution containing 1 gm. borax rotated in a 2 dm. tube +1.18° to the right; hence $[\alpha]_D^{20} = +22.09°$.

The melting point and optical rotation of this alcohol agree so closely with the physical constants recorded for volemite,[4] that the identity of the two substances might be suspected except for one discordant fact. The melting point of the benzal derivative of volemite is given by Bougault and Allard[4] as 90°, which does not agree with the melting point (200°, see below) for the benzal compound of the heptitol obtained from the sugar sirup. For the present, therefore, the alcohol will be designated as *α-sedoheptitol.*

Tribenzal-α-Sedoheptitol.—3 gm. of α-sedoheptitol were dissolved in 4 cc. of 50 per cent sulfuric acid, 4 gm. of benzaldehyde

[4] Fischer (*Ber. chem. Ges.*, 1895, xxviii, 1973) gives 149–151° as the melting point of volemite. Bougault, J., and Allard, G. (*Compt. rend. Acad.*, 1902, cxxxv. 796), record for volemite $[\alpha]_D$ in water + 1.92° and +2.65°, and in borax solution +20.83°.

were added, and the mixture was shaken thoroughly and allowed to stand over night. The crystalline product of the reaction was washed with water and then with alcohol and finally was twice recrystallized from absolute alcohol. It melted sharply and without decomposition at 199–200° (uncorrected). Analysis shows it to be the tribenzal compound of the heptitol.

0.1227 gm. substance gave 0.3174 gm. CO_2 and 0.0613 gm. H_2O.

	Calculated for $C_{21}H_{26}O_7$:	Found:
C	70.58	70.54
H	5.88	5.55

Tribenzal-β-Sedoheptitol.—The sirupy residue from the α-heptitol, together with the alcoholic washings, was dissolved in water and the solution was concentrated to a sirup. This was dissolved in an equal volume of 70 per cent sulfuric acid in a small glass-stoppered Erlenmeyer flask, and the same volume of benzaldehyde was added. On vigorous shaking crystallization began at once and after 1 hour the contents of the flask had changed to a semisolid mass of crystals. After standing over night these were thoroughly washed with water, filtered off, and then washed again with alcohol. The product was finely ground and was then boiled out several times with alcohol and twice with methyl ethyl ketone. It then melted at about 225–230° but was probably not pure as it may have contained a little of the benzal compound of the α-heptitol and perhaps other substances. It was, however, used in this condition for hydrolysis. For analysis a small part was recrystallized by dissolving in a large volume of hot methyl ethyl ketone, in which it is rather sparingly soluble, and concentrating the solution to a small volume. In all other reagents it is nearly insoluble. It separates from methyl ethyl ketone in tufts of flexible needles resembling asbestos. It melts at 272–275° (uncorrected) without decomposition and crystallizes again on cooling. Analysis shows it to be the tribenzal derivative of a second heptitol which will be named *β-sedoheptitol.*

0.1418 gm. substance gave 0.3646 gm. CO_2 and 0.0727 gm. H_2O.
0.1291 " " " 0.3320 " " " 0.0663 " "

	Calculated for $C_{21}H_{26}O_7$:	Found:	
C	70.58	70.11	70.13
H	5.88	5.69	5.70

β-Sedoheptitol.—15 gm. of tribenzal-β-sedoheptitol (m. p. 225°), which had been purified as described above by extraction with alcohol and methyl ethyl ketone, were finely powdered and boiled for about 12 hours under reflux with 250 cc. of 8 per cent sulfuric acid. When hydrolysis was complete the benzaldehyde was removed with ether and the sulfuric acid with barium carbonate. The solution yielded on evaporation a colorless sirup which crystallized after a few days standing. The mass of crystals was washed with a little 95 per cent alcohol and recrystallized by dissolving in a little water and adding hot absolute alcohol to a faint turbidity. The substance crystallized in short thick prisms which melted at 127–128° (uncorrected), and two additional recrystallizations did not change this melting point. The yield was 6 gm. The compound appears to be optically inactive, since 0.5084 gm. of substance in 5 cc. of water showed no appreciable rotation in a 1 dm. tube. The same amount of substance in 5 cc. of saturated aqueous borax solution showed a rotation of not more than +0.1°. A 20 per cent solution in saturated aqueous borax solution of material three times recrystallized showed a rotation not greater than 0.03°, from which it may safely be concluded that the substance is optically inactive. The analysis agrees with the formula of a heptitol.

0.1651 gm. substance gave 0.2390 gm. CO_2 and 0.1097 gm. H_2O.

	Calculated for $C_7H_{16}O_7$:	Found:
C	39.62	39.47
H	7.54	7.38

The hydrolysis of the benzal compound can also be effected by means of acetic acid, and since much less time is required by this method it is perhaps to be preferred to the one just described. The benzal compound was boiled with 60 per cent acetic acid under reflux until all of it had dissolved and then the solution was distilled under reduced pressure to a sirup. This was diluted with water, the remaining benzaldehyde extracted with ether, and the aqueous solution concentrated to a sirup. This crystallized readily on seeding.

A Benzal Compound from the Transformed Sirup (Dibenzal-Anhydro-Sedoheptose).

It has already been stated that when the sirup containing sedoheptose is treated with dilute hydrochloric acid, a transformation takes place which is indicated by a marked loss in its power of reducing Fehling's solution and by the development of a strong levorotation.

In order to isolate the product of this reaction the sugar sirup was treated with benzaldehyde and sulfuric acid in the hope of obtaining a crystalline benzal compound. It was found that such a derivative could indeed be prepared either from the sirup obtained by evaporating the solution of the transformed sirup or equally well from the crude sirup itself; this would not be unexpected because the sulfuric acid that is employed in the preparation of the benzal compound would naturally bring about the transformation.

60 gm. of crude sirup were mixed with an equal weight of 70 per cent sulfuric acid in a glass-stoppered Erlenmeyer flask at room temperature and 40 gm. of benzaldehyde were added. The mixture was shaken vigorously from time to time throughout the day. Crystals began to appear after 1 to 2 hours, and after 12 hours the contents of the flask had changed to a semisolid crystalline mass. After standing at least 18 hours the mass was thoroughly broken up, filtered off, and washed successively with water, cold glacial acetic acid, and alcohol. The yield was 22 gm. In another experiment, where purified sirup was used, 15 gm. yielded 7.5 gm. In this case the material had been heated in 10 per cent solution with 1 per cent hydrochloric acid and the latter then removed with silver carbonate. In a third experiment, 20 gm. of purified sirup, without previous treatment with hydrochloric acid, yielded 9 gm. of the compound.

The substance crystallizes in prismatic needles which are practically insoluble in all the usual reagents except hot acetic acid and hot acetic anhydride. Acetic acid, however, causes some decomposition. It was recovered unchanged from ten to thirty parts of hot acetic anhydride in beautiful white prisms which often attained a length of 1 cm. 22 gm. of crude material gave 20 gm. of pure substance which melted at 245° (uncorrected).

Analysis and molecular weight determination indicate that the compound has the formula $C_{21}H_{20}O_6$.

0.1380 gm. substance gave 0.3448 gm. CO_2 and 0.0665 gm. H_2O.
0.1429 " " " 0.3586 " " " 0.0696 " "

	Calculated for $C_{21}H_{20}O_6$:	Found:	
C	68.47	68.13	68.43
H	5.43	5.33	5.20

The molecular weight (M) was determined by the boiling point method, using acetic anhydride as a solvent. As K for acetic anhydride was not known this was first determined, using benzil as the standard, and was found to be about 60, as an average of a large number of determinations.

Determination of M (Calculated 368).

$$\text{I.}\quad M = 60\frac{0.2703 \times 100}{21.87 \times 0.235} = 315$$

$$\text{II.}\quad M = 60\frac{0.5470 \times 100}{21.87 \times 0.335} = 445$$

$$\text{III.}\quad M = 60\frac{0.8028 \times 100}{21.87 \times 0.625} = 350$$

$$\text{IV.}\quad M = 60\frac{0.4867 \times 100}{22.78 \times 0.402} = 318$$

$$\text{V.}\quad M = 60\frac{0.7727 \times 100}{22.78 \times 0.542} = 375$$

The formula $C_{21}H_{20}O_6$ agrees for the dibenzal derivative of a compound having the formula $C_7H_{12}O_6$; *i.e.*, a heptose ($C_7H_{14}O_7$) minus a molecule of H_2O.

Since the sugar itself before treatment with acids behaves normally when reduced with sodium amalgam and gives a heptose osazone of normal composition, it is not possible that it could have any other formula than $C_7H_{14}O_7$. It appears therefore that this normal heptose upon treatment with acids loses the elements of H_2O from its molecule and is converted into an anhydride. By hydrolysis of the benzal compound it was indeed found possible to isolate such an anhydride in pure crystalline condition.

Anhydro-Sedoheptose.

25 gm. of the dibenzal compound just described, of the formula $C_{21}H_{20}O_6$, were suspended in 150 cc. of 60 per cent acetic acid

and boiled under reflux. Complete solution took place after ½ hour. The heating was continued for 30 minutes longer, after which the solvents and the greater part of the benzaldehyde were removed by distillation under diminished pressure. The resulting sirup was taken up in water, extracted with ether, and again evaporated as before. This sirup, which was quite colorless, reduced Fehling's solution and from it a small amount of phenylosazone could be obtained. It crystallized after several days standing. The substance after washing with 95 per cent alcohol was recrystallized from the same solvent and yielded short, thick, well formed, colorless crystals which sometimes attained the length of 0.5 cm. It melted at 155° (uncorrected).

$$[\alpha]_D^{20} = \frac{-6.02^\circ \times 5.2149}{1 \times 1.020 \times 0.2149} = -143.0^\circ.$$

The yield of the first product was 9.6 gm. while 0.3 gm. crystallized from the first mother liquor.

The same substance may be obtained directly from the transformed purified sirup if crystals for seeding are available. 35 gm. of purified sirup in 160 cc. of 1 per cent hydrochloric[5] acid were heated for 1 hour on the steam bath. The solution rotated in a 2 dm. tube −28.6°, and as calculated from the specific rotation given above contained about 16 gm. of the anhydride. The hydrochloric acid was removed from the solution with silver carbonate and the slight excess of silver with hydrogen sulfide. The sirup obtained on concentration under diminished pressure was seeded with crystals obtained from the above experiment and yielded after several days standing 8 gm. of the same substance, or 50 per cent of the amount present. It was separated from the thick sirup by diluting with very little 95 per cent alcohol, filtering off the crystals, and washing with the same solvent. It was recrystallized from 95 per cent alcohol and melted at 155° (uncorrected) without decomposition.

[5] Since the sirup generally contained a small amount of barium salts, it must be neutralized to Congo before adding the required amount of hydrochloric acid.

0.1554 gm. substance gave 0.2514 gm. CO_2 and 0.0852 gm. H_2O.
0.1677 " " " 0.2680 " " " 0.0917 " "

	Calculated for $C_7H_{12}O_6$:	Found:	
C	43.75	44.14	43.58
H	6.21	6.09	6.08

0.2870 gm. substance depressed the freezing point of 17.35 gm. H_2O 0.180°.

	Calculated for $C_7H_{12}O_6$:	Found:
M	192	175

The compound will be named *anhydro-sedoheptose.*

Properties.—It showed a levorotation in aqueous solution as follows:

2.2799 gm. substance in 25 cc. H_2O at 20° in a 4 dm. tube rotated 53.39° to the left; hence $[\alpha]_D^{20} = -146.3°$. This value was more accurately determined than the previous one and is accepted as the correct specific rotation.

No mutarotation was observed. The substance is of neutral reaction and possesses a sweet taste. It is only slightly soluble in cold 95 per cent alcohol, fairly easily in the hot solvent, and very easily in cold water. It gives a strong test with orcin and hydrochloric acid. It shows no reduction of Fehling's solution, but after warming with dilute hydrochloric acid the solution becomes reducing. When 0.2335 gm. of the non-reducing anhydro compound dissolved in 20 cc. of 1 per cent hydrochloric acid was heated for 30 minutes on the steam bath in a closed vessel, the amount of reducing substance produced corresponded to 0.0859 gm. of Cu_2O (= 0.0429 gm. of glucose). A second experiment in which 0.2335 gm. was used showed a reducing power after the same treatment which corresponded to 0.0865 gm. of Cu_2O (= 0.0432 gm. of glucose). Referring back to where the action of hydrochloric acid on the sugar sirup was discussed, it was stated there that this reagent caused a change in the power of the sugar sirup to reduce Fehling's solution and that the amount of reducing power of a solution so treated was equal to about 20 per cent of that which the original solution possessed. From the experiment just described, it will be apparent that from a given amount of the non-reducing anhydro-sedoheptose an amount of reducing substance is developed on treatment with 1 per cent hydrochloric acid which, calculated as glucose, is equal to about 18.4 per cent of the amount of anhydride taken. Thus it would seem that in dilute acids about the same condition of equilibrium is reached

between the reducing and non-reducing substances (sedoheptose and anhydro-sedoheptose, respectively), regardless of which one was originally present. It is thus clear that the product obtained by cleavage of the benzal compound of anhydro-sedoheptose with acids was a solution containing both substances in the above proportions, from which a corresponding amount of phenylosazone could be obtained. Since bromine was without action on such a mixture it may be safely concluded that the reducing compound present (sedoheptose) is of the nature of a ketose. It may be assumed also that the oxygen atom of the carbonyl group is involved in the process of elimination of water from the original sugar molecule and that this is the cause of the disappearance of its power to reduce Fehling's solution. Since the heptoses ($C_7H_{14}O_7$) contain six reactive hydroxyl groups, as shown by the fact that the fully acetylated derivative of α-glucoheptose is a hexacetate, it is to be expected that an anhydroheptose ($C_7H_{12}O_6$) would contain two less; *i.e.*, four. This corresponds to the fact that anhydro-sedoheptose yields a dibenzal derivative which can be boiled with acetic anhydride without the occurrence of any acetylation, showing that no reactive hydroxyl groups remain.

SUMMARY.

An aqueous extract of the leaves and stems of one of the stonecrops, *Sedum spectabile*, was found to contain a non-fermentable reducing sugar. The analyses of the crystalline phenyl and bromophenyl osazones that were obtained from it show that the sugar is a new heptose and it has been named *sedoheptose*. From its osone a crystalline compound with *o*-phenylenediamine was obtained. By reduction of a solution of the sugar from the plant extract with sodium amalgam two heptahydroxy alcohols were produced, designated as α- and *β-sedoheptitol*. They were obtained in pure crystalline condition and likewise their benzal derivatives. On heating the plant extract with dilute acid it lost about 80 per cent of its reducing power towards Fehling's solution. When the extract that had been heated with acid was treated with benzaldehyde a crystalline compound was obtained which proved to be the benzal derivative of a heptose that had lost one molecule of water. By hydrolysis of this benzal com-

pound the anhydride was obtained as crystalline *anhydro-sedoheptose*. On heating the latter with dilute acid it passed to the extent of about 20 per cent into sedoheptose, and there is thus an equilibrium in such solutions between the two substances. Sedoheptose is in all probability a ketose, as bromine does not oxidize it and the two alcohols mentioned above probably result from its reduction.

THE CONCENTRATION OF DEXTROSE IN THE TISSUES OF NORMAL AND DIABETIC ANIMALS.

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In the investigation of the fate of dextrose in the animal organism little attention has been given to the amount of this substance existing in the tissues. The object of this paper is to present the results of a study of the distribution and concentration of dextrose or copper-reducing substances[1] in normal and diabetic animals under various conditions. Such an investigation is of importance because of the lack of reliable data in the literature, the possible bearing on the increase during fasting in the respiratory quotient of individuals with diabetes mellitus, the fact that certain diabetics apparently neither excrete nor burn large amounts of carbohydrate taken in the form of oatmeal or other starchy foods, and also because of the possible difference between normal and diabetic organisms in their ability to hold carbohydrate in the form of dextrose in their tissues.

LITERATURE.

In 1853 Claude Bernard (1) published a large number of analyses of liver tissues for dextrose from a variety of mammals, birds, reptiles, and fish. He found in mammals percentages varying from 1 to 4 per cent in all cases when estimations were possible. These tissues were extracted with water

[1] It is appreciated that the copper-reducing power of animal tissue extracts is not due solely to dextrose, but that such substances as creatine, creatinine, uric acid, etc., may play a part. On the other hand, there are substances which may inhibit copper reduction. As the method employed in estimating the reducing power of the tissue extracts was such as to reduce the error caused by these substances to a minimum, the main conclusions are probably little affected by them. The use of the word "dextrose" in connection with its concentration in body tissues signifies the total reducing power of the extract under the conditions imposed.

and enzyme action was stopped by boiling, but in most instances the tissues were not extracted until several hours after the death of the animal. Several years later lower values, 0.08 to 0.44 per cent, for dextrose in the liver were reported by Dalton (2), who appreciated the fact that the concentration of sugar in the liver increased rapidly after the death of the animal and that this increase was due to the hydrolysis of glycogen. By thrusting the livers of well nourished rabbits taken before death into boiling water von Mering (3) found only traces of dextrose. Concentrations of 0.46 to 0.55 per cent in dogs, and 0.56 to 0.60 per cent in rabbit livers were found by Seegen and Kratschmer (4). The work of these investigators was criticized by Girard (5) on the ground that too much time elapsed before the tissues were put into boiling water, also that the tissues contained too much blood. By working quickly, pressing out all possible blood, and extracting with boiling water, he failed to find more than 0.05 per cent dextrose in dog liver.

By far the largest number of tissue analyses for dextrose, 200 or more, have been made by Pavy (6). He used the reliable technique of extracting in boiling water or cold alcohol, and his results include all the important tissues in a great variety of normal animals. Unfortunately the data do not include the blood sugar of the animals from which the various tissues were taken. Pavy considered 0.2 to 0.3 per cent the amount of dextrose ordinarily found in normal dog liver, although he reports values as high as 1.9 per cent. In rabbit livers he found 0.11 to 0.26 per cent. Other tissues of the dog reported are muscle 0.2 to 0.4 per cent, spleen 0.16 to 0.20 per cent, kidney 0.11 to 0.17 per cent, pancreas 0.08 to 0.23 per cent; lungs 0.09 to 0.32 per cent, and brain 0.07 to 0.13 per cent. Butte's (7) experiments led him to the conclusion that normally there was no sugar in the muscles of dogs, but in 30 minutes after an intravenous sugar injection of 4 gm. per kilo he found as much as 0.42 per cent. Bang (8) found 0.06, 0.07, and 0.10 per cent dextrose in the muscles of normal rabbits.

Very few analyses have been made except in normal animals. Hédon (9) reports a few experiments in which the liver was analyzed for dextrose following extirpation of the pancreas in dogs. His values vary between 0.3 and 1.28 per cent, which cannot be considered reliable because he frequently took 5 to 10 minutes in bleeding the animal before taking tissues. The kidneys of six phlorhizinized dogs were found by Levene (10) to have 0.22, 0.22, 0.20, 0.55, 0.15, and 0.28 per cent of dextrose respectively. In dogs that had been made glycogen-free Embden (11) found only traces of dextrose in the liver. Bang (8) reports six experiments performed with starving rabbits in which 2 to 5 gm. of dextrose were given intravenously. The percentage of dextrose in the liver shortly after the end of the injection was found to be considerably higher than that of the blood. In animals with blood sugar of 0.22, 0.20, 0.46, 1.10, and 0.40 per cent the liver contained 0.61, 0.80, 0.85, 1.12, and 0.94 per cent dextrose respectively. Albertoni and Monetti (12) report no dextrose found in muscles of dogs and rabbits. Kleiner (13) has reported analyses of muscles of dogs, in which the aorta and vena cava had been ligated near the diaphragm, before and after intra-

venous injection of dextrose. In his experiments ether and morphia were used as anesthetics, 4 gm. of dextrose in 20 per cent solution allowed to run into the vein at the rate of about 3 cc. per minute, and the animal was killed 1½ to 2 hours after the end of the injection. Three such experiments are reported. Blood sugars just before injection were 0.33, 0.15, and 0.17 per cent, muscle sugars before injection 0.26, 0.40, and 0.52 per cent, after injection 0.36, 0.71, and 0.80 per cent respectively. He also found a considerable increase in the reducing power of the muscle extracts after boiling with 1.8 per cent hydrochloric acid, especially after injection of sugar. This was most marked when sugar was injected into dead animals. In three of his experiments the increase amounts to as much as the sugar found before hydrolysis. The muscle tissue after being passed through a meat chopper was extracted with water under toluene for 20 hours in the ice chest. After filtering through cheese-cloth a little acetic acid was added to the extract, which was then boiled and filtered through glass wool, the final clearing of proteins being accomplished by phosphotungstic acid.

From the foregoing review of the literature, a wide variation is seen to exist in the amounts of dextrose found in animal tissues. With the exception of Pavy's (6), most work has been done with the liver, which in dogs is credited with concentrations of dextrose varying between traces and 4 per cent, while in rabbits amounts from mere traces to 0.26 per cent are given. In dogs the muscle sugar values vary between traces and 0.40 per cent. The amounts found in rabbit muscles vary between traces and 0.10 per cent. The concentrations of dextrose in spleen, pancreas, kidney, lungs, and brain, as given by Pavy, range between 0.08 and 0.13 per cent with little difference between the several organs.

The chief criticism of the work on dextrose in animal tissues is the unreliable technique employed. In most instances the rapidity with which hydrolysis of glycogen may occur did not seem to be appreciated. Only a few workers have included the blood sugar values of the experimental animals. Also, comparisons between diabetic and non-diabetic conditions have never been made.

Analyses of human tissues, both normal and diabetic, were made by Griesinger (14), but the tissues were taken several hours after death so that the results are of little value.

EXPERIMENTAL.

Much experimentation was carried out to determine the most efficient and reliable method for extraction of dextrose from animal tissues. A very essential detail which has apparently been overlooked in previous work is the prevention of enzyme action at the time of taking tissues for analysis. Boiling water and boiling sodium sulfate solution acidified with acetic acid, boiling and cold (−5–10°) alcohol, as well as the use of liquid air

were all tried as methods of preventing enzyme action and also to test their relative practicability. Boiling water kills very quickly all enzymes found in the several tissues, while alcohol, especially cold alcohol, and liquid air merely inhibit their action so that the danger in future manipulation of getting some enzyme action is not inconsiderable. Acid mixtures were discarded because of the danger of possible hydrolysis of higher sugars and sugar compounds.

Tissues ground in a meat chopper before extraction were found to increase the sugar content to double or treble the amount found if enzyme action were stopped immediately on removal of the tissues from the body, thus prohibiting this procedure. Extraction in the cold with water proved unsatisfactory because enzyme action was not wholly inhibited by temperature above 0°C.

Boiling water proved to be more efficient than alcohol in checking enzyme action; hence it was adopted. The difficulty in obtaining and manipulating liquid air, with its slight superiority over boiling water, led us to discard its use.

The manner in which protein could be removed from the extracts was the subject of considerable investigation. A distinct advantage offered by alcohol is that the protein is taken out in the process of extraction, while in salt or acidified solutions only a portion of the protein is taken out, necessitating a second procedure for its complete removal. After experimenting with meta-phosphoric acid, phosphotungstic acid, hydrochloric acid, and mercuric chloride (Schenk's method), aluminium hydroxide, and kaolin, colloidal iron (Michaelis and Rona (30)) was found to be the most convenient and satisfactory.

Method.

The experiments were performed on dogs and rabbits. Except in two or three particular instances anesthesia of any kind was avoided because of the well known effect on blood sugar and the possible alterations in the various tissues which might follow. In rabbits, intravenous injections were made in the ear vein, and blood samples were taken from the heart. In dogs, intravenous injections were made and blood samples taken from the external jugular vein by means of a Luer syringe. Before taking tissues

for analysis all animals were stunned by a blow on the head, and, with the exception of a few animals which were bled from the carotid for 3 or 4 minutes, the tissues were immediately weighed on an Eimer and Amend No. 330 triple beam balance and thrust immediately into boiling water. The sample was then cut into small pieces in the boiling water, boiling being continued for at least 15 minutes. At the end of this time the beakers were placed on a boiling water bath. As soon as convenient the water covering the tissue was filtered through glass wool and the tissue finely divided with a macerator. A second extraction on the water bath for a period of 1 hour was made, and finally three such extractions of 10 minutes each, making altogether five water extractions. The combined extracts which had been filtered through glass wool were measured (usually about 700 to 800 cc. for 50 gm. of tissue) and the protein was removed by colloidal iron (Merck's 5 per cent). It was found that muscle extracts require 2 cc. per gm. of tissue, while all other extracts cleared perfectly with 1 cc. of colloidal iron per 1 gm. of substance. The iron is added slowly with constant stirring or shaking, 5 to 10 cc. of a 40 per cent sodium sulfate (crystals) solution are added in a similar manner and placed on the water bath for an hour, and then filtered.[2]

The entire or an aliquot part of the filtrate is concentrated to small volume on the water bath. All liver extracts, however, were concentrated *in vacuo* because of the tendency of these extracts to become highly colored when brought to a small volume in the water bath. Benedict's (15) titration method for sugar estimation was used because creatine, uric acid, etc., cause less error than in Fehling's solution. Frequently only small amounts of material were available, so 10 cc. instead of 25 cc. of the copper solution were used and in instances where there was not sufficient to reduce the entire amount of copper the titration was finished by adding the necessary amount of a 0.2 per cent dextrose solution. Blood sugar was determined by the method of Lewis and Benedict (16).

[2] Both sodium sulfate and heating are not absolutely essential, but we found that they facilitated filtration considerably. The use of colloidal iron for removal of protein from solutions containing dextrose was suggested by Michaelis and Rona (30).

Normal Animals.

Dogs were taken from the stock supply (ordinary diet, bread and soup) and on the day of the experiment received no food. The results of the analyses are collected in Table I.

TABLE I.

Normal Dogs.

No. of experiment.	Blood sugar.		Dextrose in tissues.										
	Whole blood.	Plasma.	Liver.	Skeletal muscle.	Heart muscle.	Kidney.	Spleen.	Pancreas.	Stomach.	Small intestine.	Skin.	Lungs.	Brain.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
N IV	0.10	0.11	0.26	0.07	0.20				0.04				
			(0.27)*	(0.10)*	(0.25)*				(0.04)*				
342**	0.10	0.10	0.47	0.04	0.07	Trace.	0.06	0.06	0.03	0.02		0.01	0.01
341**	0.11	0.12	0.22	0.07	0.02	0.05	0.02		0.01	0.03	0.01	0.01	0.02
343**	0.11	0.13	0.43	0.07		0.05	0.03	Trace.	0.02	0.01	0.02	0.04	0.01
316†	0.13	0.10	0.14	0.09	0.07	0.07	0.07	0.11	0.04	0.04	Trace.	0.06	Trace.
321‡				0.07	0.05	0.07							0.05

* After hydrolyzing extracts with 1.8 per cent HCl, and boiling for 1½ hours with a reflux condenser.

** Animals were bled before taking of tissues.

† Dog proved to have a tumor of the ovary. Tissues in addition to those in the table showed: bladder 0.04 per cent; parotid 0.04 per cent; esophagus 0.03 per cent; ovarian tumor 0.03 per cent; tongue 0.02 per cent; thyroid, aorta, and trachea only traces.

‡ Died under ether anesthesia; tissues were taken within 10 minutes.

The blood sugar in these animals varied between 0.10 and 0.13 per cent, averaging 0.11 per cent, which is within the range usually given for the normal blood sugar values in dogs (16). A wide variation in the liver dextrose is at once noticeable, varying from 0.14 to 0.47 per cent, making any average of the several observations of little significance. The concentration of dextrose in the muscles is well under that found in the blood or liver and is much more constant, varying from 0.04 to 0.09 per cent, averaging 0.07 per cent. The heart muscle with one exception, N IV, seldom contained more sugar than did the skeletal muscle. In the other tissues, kidneys, spleen, pancreas, stomach, intestine, skin, tongue,

thyroid, and brain, the amount of dextrose present is small and with the exception of the pancreas in Dog 316 invariably lower than in the muscle. All of these tissues taken together, of course, amount to much less than the muscle, which is usually considered to comprise three-sevenths of the body weight. In Dog N IV a slight increase in the dextrose in the muscle extracts is found after boiling with hydrochloric acid. The skeletal muscle increased from 0.07 to 0.10 per cent, the heart muscle from 0.20 to 0.25 per cent, but no increase was found in either liver or stomach.

Effect of the Duration of Hyperglycemia.

Before making observations on animals in which dextrose had been administered to determine the amount of sugar the several

TABLE II.

Effect of the Duration of Hyperglycemia on the Concentration of Dextrose in Normal Rabbits.

No. of experiment.	Dextrose in plasma before sugar injection.	Dextrose in blood immediately before killing.		Dextrose in tissues.			Remarks.
		Whole blood.	Plasma.	Liver.	Muscle.	Stomach.	
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
I	0.13			0.09			Received no dextrose.
II	0.17			0.17			" " "
III	0.16	0.35	0.50	0.37	0.11	0.12	Received 8 gm. of dextrose per kilo subcutaneously; killed 2½ hrs. after sugar injection.
IV	0.13	0.44	0.50	0.42	0.08	0.12	Received 8 gm. of dextrose per kilo at beginning of experiment; 8 hrs. later 4 gm. per kilo given subcutaneously. Killed 12 hrs. after first injection.
V	0.20	0.42	0.49	0.44	0.04	0.21	Received 8 gm. of dextrose per kilo at beginning; 12 hrs. later 4 gm. per kilo and 22 hrs. later 4 gm. per kilo. All sugar was given subcutaneously. Killed 24 hrs. after first injection.

Merck's anhydrous dextrose was used in all experiments including the use of dextrose.

tissues had the power of taking up, experiments were performed to see what effect the duration of hyperglycemia might have in this respect. Rabbits were chosen for this purpose. The results are given in Table II.

A hyperglycemia maintained over periods from 2½ to 24 hours had little effect on the amount of sugar that the liver, skeletal muscle, and stomach held as free dextrose.

Effect of Bleeding on Dextrose in the Tissues.

In certain of the experiments a large quantity of blood was desired for special investigations. Hence bleeding from the carotid for 3 to 4 minutes was performed until from an animal weighing between 10 and 15 kilos 400 to 500 cc. of blood were taken. The impression gained from the literature was that glycogenolysis in the liver to any marked extent did not take place for 2 or 3 minutes after the death of the animal. We anticipated that if the tissues were taken before the heart stopped beating there would be no significant change. It soon developed that bleeding had a profound effect on glycogenolysis in the liver. At the same time it came to light that glycogenolysis in the muscles and other tissues did not occur to any significant extent, making it possible to study the effect of bleeding on the liver dextrose and at the same time to use the other tissues for dextrose determinations. The liver tissue was taken immediately after the animal was stunned, thus avoiding the effect of subsequent bleeding.

If the liver tissue be taken immediately (within 10 seconds) after stunning, before bleeding has progressed to any extent, there is apparently little hydrolysis of glycogen, as may be seen in Experiments 351, 355, 352, and 353. Plasma and liver dextrose correspond quite closely except in Dog 350, where the difference is considerable. As the time of taking the specimen increases, so also does the dextrose in the liver tissue until in one instance, Dog 344, when 5 minutes elapsed from the time the animal was stunned, but only 1 minute from the end of the bleeding, before the liver was taken, an increase of the tissue over the plasma sugar amounting to 340 per cent had occurred. When the animals were not bled but tissue was taken soon after stunning, out of five animals in only one did the liver contain much more dextrose than the

plasma. The increase in dextrose in consecutive specimens of liver was found to be greater in the animals which were bled than in those not bled. In diabetic animals, as might be expected when

TABLE III.

Effect of Bleeding on Dextrose in the Liver.

No. of experiment.	Plasma dextrose.	Time between stunning and taking of liver.	Liver dextrose.	Difference between plasma and liver.
		Normal animals bled.		
	per cent		*per cent*	*per cent*
350	0.27	10 sec.	0.37	+0.10
351	0.28	10 "	0.28	0.00
355	0.83	10 "	0.75	−0.08
352	1.06	10 "	0.75	−0.31
353	0.08	1 min.	0.08	0.00
347	0.19	2 "	0.85	+0.66
342	0.10	4 "	0.48	+0.38
341	0.12	5 "	0.22	+0.10
344	0.13	5 "	0.58	+0.45
343	0.13	6 "	0.43	+0.30
340	0.21	7 "	0.80	+0.59
		Normal animals without bleeding.		
315	0.55	10 sec.	0.47	−0.08
317	0.27	1 min.	0.32	+0.05
316	0.10	1 "	0.14	+0.04
297	0.68	2 "	0.65	−0.03
325	0.26	3 "	0.47	+0.21
		Diabetic animals bled.		
302	0.53	4 min.	0.34	−0.19
319	0.44	5 "	0.20	−0.24
322	0.56	7 "	0.38	−0.18
328	0.71	8 "	0.35	−0.36
		Diabetic animals without bleeding.		
280	0.26	10 sec.	0.26	0.00
271	1.00	1 min.	0.54	−0.46
287	0.29	1 "	0.16	−0.13
320	0.58	7 "	0.49	−0.09

glycogen in the liver exists in small amounts or is absent, the liver dextrose is invariably lower than the plasma. Glycogenolysis in muscles is much slower than in the liver. This was shown by repeated analyses of muscle samples taken at intervals during 15 minutes after stunning. The changes were always insignificant.

Intravenous Dextrose in Rabbits.

Bang (8) has reported a series of experiments on normal fasting rabbits in which following an intravenous injection of dextrose there was a higher concentration of sugar in the liver than in the blood. This observation seemed of sufficient importance to confirm because of its bearing on glycogenesis and its possible significance in diabetes. Accordingly Bang's experiments were repeated. Normal rabbits which had been fasted for 6 days were used (Table IV).

TABLE IV.

Intravenous Dextrose in Rabbits. Effect on Dextrose in Liver.

No. of experiment	VI.	VII.	VIII.	IX.	X.	XI.
Weight, *gm*	1,750	2,000	2,750	2,175	2,125	1,925
Amount of dextrose injected, *gm*	2.0	2.0	4.0	4.0	5.0	5.0
Time for injection, *min*	13	13	15	15	21	30
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Blood sugar before injection	0.14	0.13	0.13	0.15	0.13	0.15
" " after "	0.38	0.41	0.56	0.72	0.67	1.06
Plasma " " "	0.56	0.54	0.72	0.93	0.98	1.47
Liver dextrose	0.40	0.41	0.54	0.66	0.67	0.72
Muscle "	0.21	0.12	0.17	0.18	0.16	0.20
Liver glycogen	Trace.	Trace.	0.25	0.31	0.53	1.03
Muscle "	"	"	0.08	0.10	0.15	0.13

We were not able to confirm Bang's observations, for in all six rabbits the concentration of sugar in the liver was below that in the plasma, and also below the whole blood values except in Rabbit VI, where the difference amounts to 0.02 per cent with a whole blood sugar of 0.38 per cent. From a trace in the liver, when 2.0 gm. of dextrose were given, the glycogen increases to 0.53 and 1.03 per cent in the two rabbits receiving 5.0 gm. Both dextrose and glycogen exist in small amounts in the muscles.

Effect of Dextrose Administration in Normal Animals.

Dextrose was given by mouth, subcutaneously, intravenously, and intraperitoneally to determine, in addition to the effect of hyperglycemia itself, whether the manner of administration played any part in the ability of tissues to take up dextrose. That there might be a difference in the behavior of tissues in concentrating sugar was suggested by the work of Allen (17) who found that after administration of dextrose to normal animals by any route except intravenously, a marked oliguria resulted, whereas sugar given to diabetic animals by any route whatsoever caused a marked diuresis. Allen's interpretation of this phenomenon was that in normal animals the sugar in passing through the various tissues to the blood stream when given by mouth, subcutaneously, or intraperitoneally underwent some change which resulted in its appearance in the blood in a different physiological state than when introduced intravenously. The diuresis, glycosuria, and loss of power of utilization of sugar in diabetes are thought to be due in part to the presence of dextrose in the blood in some abnormal (probably crystalloid) state.

In all the experiments with the exception of Dog I II the attempt was made to kill the animal at the height of the hyperglycemia. Four animals were given levulose by mouth to see if this particular sugar was held in amounts different from dextrose. The results of the several experiments are collected in Table V.

As in all other experiments, the chief interest centers about the dextrose in the muscles and the liver. It will be seen that the amounts of dextrose found in the liver are quite variable. The extreme, and apparently somewhat variable, rapidity with which glycogenolysis occurs makes the liver values less significant than was hoped. When compared with normal values there is an increase in the muscle dextrose as the blood sugar increases, but it is not always directly proportional to it.

In the animals receiving sugar by mouth, having a plasma dextrose of 0.24 and 0.63 per cent, there was 0.11 and 0.21 per cent respectively in the skeletal muscles. When large amounts of sugar were given subcutaneously the plasma sugar varied between 0.25 and 0.40 per cent but the skeletal muscle dextrose never rose above 0.10 per cent. The highest values were obtained after intravenous administration of dextrose. Dog 352 had a plasma sugar

TABLE V.

Normal Dogs. Dextrose by Mouth, Subcutaneously, Intravenously, and Intraperitoneally.

No. of experiment.	Amount of sugar given per kilo.	Time after sugar administration that tissues were taken.	Blood sugar.				Dextrose in tissues.										
			Before sugar administration.		At time the tissues were taken.		Liver.	Skeletal muscle.	Heart muscle.	Kidney.	Spleen.	Pancreas.	Stomach.	Skin.	Lungs.	Brain.	Eyes.
			Whole blood.	Plasma.	Whole blood.	Plasma.											
Dextrose by mouth.																	
	gm.	*hrs.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
315	12	1	0.18	0.20	0.55	0.63	0.47	0.21	0.08			0.12		0.10			
350	7	1	0.09	0.13	0.19	0.24	0.37	0.11	0.12	0.14	0.08	0.08		0.03	0.09	0.05	0.08
							(0.37)*	(0.20)		(0.15)					(0.09)		
Levulose by mouth.**																	
340	12	$2\frac{1}{2}$	0.10	0.11	0.19	0.21	0.80	0.06	0.05	0.09	Trace.	0.03		0.05		Trace.	
344	12	2	0.11	0.14	0.13		0.58	0.07	0.12	0.18	0.08	0.25		0.18	0.12	0.03	0.07
347	12	2	0.10	0.11		0.19	0.86	0.11	0.13	0.17	0.08	0.10		0.14	0.14	0.04	0.08
							(0.85)	(0.16)	(0.17)	(0.17)	(0.09)	(0.14)		(0.14)	(0.14)	(0.06)	
351	12	2		0.10		0.17	0.28	0.12									

Dextrose subcutaneously.

317	10	$2\frac{3}{4}$	0.09	0.09	0.20	0.27	0.32	0.10	0.22	0.20***	0.07	0.04	0.08			0.02	
333†	10	4	0.10	0.12	0.22	0.25		0.09	0.29	0.18***	0.05	0.06	0.07	0.10	0.09	0.06	
337‡	10	3	0.12	0.13	0.27	0.40	0.62	0.10	0.08	0.19§	0.05	0.07	0.08	0.03		0.03	

Dextrose intravenously.

352	55	(6 min.)		0.13		1.05	0.75	0.41	0.56				0.52	0.45	0.50	0.11	0.17
							(0.77)	(0.46)	(0.61)				(0.48)	(0.46)	(0.54)		
I II	4	$1\frac{1}{2}$	0.16	0.17	0.20	0.25	0.56	0.27	0.31				0.13				0.17
							(0.58)	(0.35)	(0.35)				(0.16)				

Intraperitoneal dextrose.

355	8	1		0.10		0.83	0.75	0.33	0.33				0.46		0.34		
							(0.72)	(0.32)	(0.36)				(0.46)		(0.34)		

* All values enclosed in parentheses are those obtained after hydrolysis with 1.8 per cent hydrochloric acid by boiling $1\frac{1}{2}$ hours with a reflux condenser.

** Levulose obtained from Schering and Glatz contained 91 per cent levulose and 9 per cent dextrose. All animals receiving levulose were bled before taking tissues. The time of bleeding did not exceed 4 minutes.

*** Urine contained about 5.0 per cent dextrose, which probably accounts for the high percentage of dextrose found in the kidney tissue.

† Tissues in addition to those in the table: tongue 0.11 per cent; esophagus 0.18 per cent; bladder 0.24 per cent; thyroid 0.03 per cent; aorta 0.13 per cent.

‡ Animal bled to death.

§ Urine contained 1.0 per cent dextrose.

of 1.05 per cent and a skeletal muscle sugar of 0.41 per cent. The sugar in heart muscle varies in much the same manner as it does in skeletal muscle and seems to have about the same concentration. Little comment is necessary concerning the various other tissues examined. Except when sugar was given by mouth the stomach was analyzed for sugar for the purpose of comparing smooth with striated muscle. Here again the higher the blood sugar the greater the amount of dextrose found in the tissue. No appreciable difference is to be noted between the effects of levulose and glucose in the concentration of sugar in the liver, muscles, or other tissues.

In several of the experiments the extracts of some of the tissues were hydrolyzed with hydrochloric acid. Only the extracts of striated muscle showed any increase in reducing power after boiling with hydrochloric acid.

Influence of Adrenalin.

Adrenalin was used to produce a hyperglycemia in normal animals to determine the effect of glycogenolysis on the tissue dextrose (Table VI).

TABLE VI.

Normal Dogs. Effect of Adrenalin.

No. of experiment.	Time after adrenalin that tissues were taken.	Blood sugar before adrenalin.		Blood sugar at time tissues were taken.		Dextrose in tissues.					
		Whole blood.	Plasma.	Whole blood.	Plasma.	Liver.	Skeletal muscle.	Heart muscle.	Stomach.	Skin.	Eyes.
	hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
361*	2		0.12		0.32	0.29	0.25	0.21	0.11		0.13
						(0.27)**	(0.24)	(0.20)	(0.12)		
A II	2¼	0.11	0.11	0.26	0.36	0.52	0.21	0.22	0.14	0.18	0.12
						(0.52)	(0.36)	(0.25)	(0.17)	(0.19)	
A III	2	0.10	0.11	0.24	0.29	0.51	0.14	0.23	0.15	0.17	0.10
						(0.56)	(0.28)	(0.27)	(0.17)		

* Parke, Davis and Company adrenalin solution 1: 1,000, 1 cc. per kilo was used in all these experiments.

** All values enclosed in parentheses are those obtained after hydrolysis with 1.8 per cent hydrochloric acid by boiling for 1½ hours with a reflux condenser.

There seems to be no striking difference between the amount of dextrose in the tissues when hyperglycemia is caused by adrenalin and when it results from the administration of dextrose. There is no elevated concentration in the tissues to indicate glycogenolysis or the pouring of dextrose by the cells into the blood. On hydrolysis the only extracts yielding extra sugar are those of skeletal muscles. In Dog A III, with a skeletal muscle sugar of 0.14 per cent, on boiling with acid the concentration is increased to 0.28 per cent.

Effect of Phlorhizin and Adrenalin.

An animal was phlorhizinized to see if any effect on the tissue sugar resulted from the rapid loss of glycogen without hyperglycemia. In two other dogs phlorhizin and adrenalin were given as recommended by Sansum and Woodyatt (18) to secure glycogen-free animals for the administration of dextrose. The purpose of these experiments was to see if the presence of glycogen in muscle bears any relation to the sugar in the tissue before and after hydrolysis. Table VII contains the results of these experiments.

It is not surprising to find in the phlorhizinized animal, No. 353, low values for dextrose in the tissues. With a decrease in glycogen the possibility of sugar increase as the result of rapid glycogenolysis during extraction is ruled out. The liver dextrose amounting to only 0.08 per cent is the lowest value found in any of the animals. No marked difference from the normal animals is noted in the thigh muscle, heart, or stomach sugar. An increase from 0.08 to 0.12 per cent in the skeletal muscle extract was found after hydrolysis. There is no marked difference between the concentration of dextrose in the tissues of Dogs P II and P III and other animals not free from glycogen. The liver values approximate those of the plasma, while thigh, heart, and stomach muscle shows amounts near the average usually found when the blood sugar is of the same relative value.

In Dog P II we started with an animal whose muscles were glycogen-free, as shown by analysis before sugar was given, nor was there any glycogen found in either muscles or liver 1½ hours after sugar was given. The dextrose in the muscles before sugar injec-

TABLE VII.

Phlorhizinized Animals.

Experiment.		Blood sugar.				Tissue dextrose.						
No.	Description.	Before sugar injection.		After sugar injection. Just before killing.		Liver.	Skeletal muscle.	Heart muscle.	Stomach.	Eyes.	Pancreas.	Lungs.
		Whole blood.	Plasma.	Whole blood.	Plasma.							
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
353	1 gm. of phlorhizin in olive oil subcutaneously 3 days in succession previous to killing for tissues. No sugar given.	0.08	0.08			0.08	0.08 (0.12)*	0.10	0.04	Trace.	0.03 *	0.03
P II**	1 gm. of phlorhizin subcutaneously and 1 cc. adrenalin 1:1,000 subcutaneously every 3 hours (nights excepted) until D:N ratio was constant. 4 gm. per kilo dextrose intravenously; killed 1½ hrs. after.	0.07	0.09	0.26		0.23 (0.27) †	0.13 (0.32) †	0.21 (0.29) †	0.15 (0.19) †	0.12		
P III**	Animal made glycogen-free as in previous experiment, 4 gm. per kilo intravenously; killed in 1½ hrs.	0.06	0.09	0.37	0.47	0.55 (0.57) ‡	0.25 (0.29) ‡ ‡	0.22 (0.27) ‡ ‡	0.15 (0.20) ‡ ‡	0.13		

* Value in parentheses after boiling with 1.8 per cent HCl.

** The sugar injections were made and first specimens of muscle taken under light ether anesthesia.

† Values in parentheses after boiling with 1.8 per cent HCl. Muscle analyzed for glycogen before sugar injection was negative. A trace was found after sugar was given. Liver negative. Sugar in muscle before sugar was given = 0.12 per cent.

‡ Values after hydrolysis are those in parentheses. Muscle analyzed for glycogen before sugar was given was found negative. Sugar content was 0.18 per cent, after hydrolysis 0.19 per cent. Liver glycogen after sugar was 0.163 per cent and muscle glycogen 0.340 per cent.

tion was 0.12 per cent, afterward 0.13 per cent, showing practically no change. However, on hydrolysis of the muscle extract after sugar injection there was an increase from 0.13 to 0.32 per cent or nearly threefold in the sugar values. Hydrolysis of the muscle extracts produced less increase in the sugar values in Dog P III. Starting with practically no hydrolyzable sugar before dextrose was given, 1½ hours afterward the skeletal muscle showed only 0.04 per cent, the heart muscle 0.05 per cent, and the stomach 0.05 per cent increase after boiling with hydrochloric acid. Although in Dog P III no glycogen was found in the muscles before sugar injection, we found 0.16 per cent and 0.34 per cent in liver and muscle respectively after injection.

Diabetic Animals without Sugar Administration.

Various stages and degrees of diabetes[3] are represented in this group of animals. A brief history of the clinical course of the dogs is found in the table giving the results of the tissue analyses (Table VIII).

The striking fact revealed in the tissue analyses of this group of animals is the low concentration of dextrose found in both liver and muscle as compared with the hyperglycemia at the time the tissues were taken. With an average plasma sugar of 0.41 per cent, the average liver sugar was 0.46 per cent, while the muscle sugar averaged only 0.12 per cent, which exceeds but little the skeletal muscle in normal animals in Table I where the plasma sugar average was only 0.11 per cent. The heart muscle is also relatively low in dextrose. In Dog 338 where total pancreatectomy was performed the plasma sugar was 0.71 per cent, the liver was only 0.35 per cent, skeletal muscle 0.14 per cent, and heart muscle 0.10 per cent. The sugar concentration in all other tissues of this series is low. Attention should be called to the fact that the tissues of diabetic animals are low in glycogen. In the livers of Dogs 280 and 322, which were in an extremely weak state, no glycogen was found in either muscles or liver. The

[3] All pancreatectomy operations were performed by Dr. F. M. Allen. Many of the diabetic dogs had been under his observation for several months.

TABLE VIII.

Diabetic Animals without Sugar Administration.

No. of experiment.	Description of animal.	Blood sugar.		Tissue dextrose.											Remarks.
		Whole blood.	Plasma.	Liver.	Skeletal muscle.	Heart muscle.	Kidney.	Spleen.	Stomach	Small intestine.	Skin.	Lungs.	Brain.	Eyes.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
300	4 months previously $\frac{9}{10}$ of pancreas was removed. Would show sugar on bread, not meat, without glycosuria. For several weeks, hyperglycemia.	0.21	0.23	0.59	0.09	0.06	0.06		0.04		0.10		0.03	0.05	
280	1½ yrs. previously $\frac{4}{5}$ of pancreas was removed. Sugar-free for 1 yr. Then given high carbohydrate diet to impair tolerance. Preceding 6 months gradually lost tolerance; showed sugar on lung and suet; developed coma; died.	0.14	0.26	0.26	0.06	0.04	0.08				0.01		0.03		Liver and muscle contained no glycogen.

320	$\frac{1}{2}$ of pancreas was removed 4 months ago. After 2 months' feeding with bread and soup showed large amounts of sugar. Sugar-free on lung and suet. Heavy glycosuria at death.	0.58		0.49	0.12	0.13	0.37	0.09	0.13	0.08	0.08	0.20	0.03	0.21	
322	$\frac{1}{2}$ of pancreas was removed 6 months ago. Excreted sugar on bread and soup. 1 month before began to emaciate; would not eat; glycosuria.		0.56	0.37	0.26	0.28			0.29						No change in sugar content after boiling with 1.8 per cent HCl. Muscles and liver contained traces only of glycogen.
319	$\frac{3}{4}$ of pancreas was removed 5 months ago. Could not take bread and soup without glycosuria. 1 month excreted sugar on lung diet. Used for several sugar administration experiments. Hyperglycemia without glycosuria.		0.44	0.20	0.02	0.08	0.12		0.12		0.01	0.12	Trace.		Heavy meat diet 3 days before death.

TABLE VIII—*Concluded.*

No. of experiment.	Description of animal.	Blood sugar.		Tissue dextrose.										Remarks.
		Whole blood.	Plasma.	Liver.	Skeletal muscle.	Heart muscle.	Kidney.	Spleen.	Stomach.	Small intestine.	Skin.	Lungs.	Brain.	Eyes.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
292	About ½ of pancreas was removed 5 months ago; the rest placed under skin. All pedicles cut 2 months ago. Glycosuria developed 1 month ago on lung and suet. Progressive.		0.27	0.83	0.05	0.15	0.10		0.09	0.05	0.06		0.06		Bled before tissues were taken.
338	Total pancreatectomy 24 hrs. before killing. Heavy glycosuria.	0.57	0.71	0.35	0.14	0.10	0.33	0.14	0.15		0.28		0.04		
397	⅝ of pancreas was removed 5 months ago. For past 2 months forced fat and protein diet without glycosuria but increasing hyperglycemia and gain in weight. Gradual coma for past 3 days; dying.		0.38	0.59 (0.59)	0.21 (0.30)										Muscle, glycogen-free. Liver, glycogen a trace. Value in parentheses = after boiling with 1.8 per cent HCl.

tissue extracts in Dog 322 showed no increase in reducing substances on hydrolysis while in Dog 397 the muscle showed an increase of 0.09 per cent with no change in the liver extract.

Effect of Sugar Administration in Diabetic Animals.

Much the same type of animal was used in these experiments as in the preceding group. It comprises mild diabetes, total pancreatectomy, and various intermediate stages (Table IX).

As in the group of diabetic dogs which were given no sugar, the striking feature is the low concentration of dextrose in the liver, thigh, and heart muscles as compared with the degree of hyperglycemia produced. The average plasma sugar was 0.72 per cent, liver 0.55 per cent, skeletal muscle 0.18 per cent, and the heart muscle 0.21 per cent. In normal animals the tissues contained a higher concentration of dextrose when the plasma sugars were comparable. The spleen, stomach, skin, lungs, brain, etc., all contain small amounts of sugar. As the plasma sugar increases there is a corresponding increase in the dextrose values of all these tissues. In Dogs 287, 297, and 309 a great variety of tissues were analyzed. The variations in amounts of dextrose in the several tissues follow quite closely the variations in hyperglycemia. No increase in sugar or hydrolysis is observed in any of the tissues of Dog 271, while in the totally depancreatized Dog D I the muscle extract nearly doubled in reducing power after boiling with acid.

Comparison of Normal and Diabetic Animals.

Both normal and diabetic tissues exhibit a tendency to increase in concentration of dextrose as the sugar in the blood increases. This tendency, however, appears to be much more marked in the normal than in the diabetic animals. To show this relation more clearly Table X has been constructed. The concentrations of dextrose in the skeletal and heart muscle of normal and diabetic animals having nearly comparable blood sugars are placed together.

No marked difference is to be seen in the liver comparisons, but both skeletal and heart muscle show a decidedly lower amount of dextrose in the diabetic than in the normal animals, when the blood sugars are comparable.

TABLE IX.

Diabetic Animals with Sugar Administration.

No. of experiment.	Description of animal.	Blood sugar.				Tissue dextrose.										
		Before sugar.		After sugar and just before death.		Liver.	Skeletal muscle.	Heart muscle.	Kidney.	Spleen.	Stomach.	Small intestine	Skin.	Lungs.	Brain.	Eyes.
		Whole blood.	Plasma.	Whole blood.	Plasma.											
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
302	$\frac{1}{5}$ of pancreas was removed 5 months ago. Tolerance decreased until at time of experiment dog has marked glycosuria on fat and protein diet. Given 1 gm. of dextrose per kilo by mouth; killed 2 hrs. later.		0.39		0.53	0.34	0.05	0.18	0.24		0.12	0.05	0.18	0.16	0.01	0.20
287**	$\frac{4}{5}$ of pancreas was removed 4 months ago. Could take high carbohydrate diet without showing glycosuria. Moderate hyperglycemia. 10 gm. per kilo subcutaneously. Died suddenly 2 hrs. later. Tissues taken.	0.17	0.18	0.27	0.29	0.22	0.05	0.11	0.22	0.09	0.09	0.03	0.06	0.17	0.04	0.10

271	$\frac{5}{6}$ of pancreas was removed $1\frac{1}{2}$ yrs. ago. By feeding, tolerance for sugar gradually lowered until the dog excreted large amounts of sugar on fat and protein diet. 0.5 gm. of dextrose intravenously and 7 gm. of dextrose subcutaneously 4 hrs. before killing.	0.33	0.40		1.00	0.48 (0.48)*	0.33 (0.32)	0.27 (0.27)	0.85 (0.82)	0.28	0.37 (0.37)	0.36 (0.35)	0.56 (0.56)	0.49 (0.45)	0.08	0.39
309***	$\frac{2}{3}$ of pancreas was removed 6 days before experiment. Dog refused to eat; began to decline with heavy glycosuria. Remnant removed at beginning of experiment. 1 gm. of dextrose per kilo intravenously 9 hrs. before death. 1 liter of salt solution 6 hrs. before death. Blood sugar before salt = 1.00 per cent.	0.17	0.20	0.67	0.80	0.83	0.39		0.39	0.34		0.18		0.33	0.10	0.39
325	$\frac{3}{4}$ of pancreas was removed 1 month before; did not do well. $\frac{1}{2}$ hr. before killing $\frac{1}{2}$ gm. of dextrose per kilo intravenously. Urine contained no sugar at beginning of experiment.	0.11	0.15	0.23	0.29	0.40	0.07	0.14	0.07	0.06			0.11		0.04	

TABLE IX—*Concluded.*

No. of experiment.	Description of animal.	Blood sugar.				Tissue dextrose.										
		Before sugar.		After sugar and just before death.		Liver.	Skeletal muscle.	Heart muscle.	Kidney.	Spleen.	Stomach.	Small intestine.	Skin	Lungs.	Brain.	Eyes.
		Whole blood.	Plasma.	Whole blood.	Plasma.											
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
297†	¾ of pancreas was removed 4 months ago. Did well up to 1 month before experiment, on bread and soup. Then began to lose weight and look sick. ½ hr. before death 0.75 gm. of dextrose per kilo intravenously. Urine contained no sugar at beginning.	0.14	0.18	0.37	0.68	0.65	0.13	0.33	0.77	0.73	0.25	0.22	0.24	0.32	0.05	
D_1‡	Total pancreatectomy 3 days before; D:N ratio before experiment 2.00. Intravenous sugar 4 gm. per kilo 1½ hrs. before tissues taken.	0.31	0.40	1.15	1.43	0.95 (0.95)	0.25 (0.47)	0.45 (0.57)			0.56 (0.53)					0.52

* All values in parentheses are obtained after boiling with 1.8 per cent hydrochloric acid for 1½ hours with a reflux condenser.

** Additional tissues: pancreas remnant 0.08 per cent; testes 0.08 per cent; thyroid 0.03 per cent; trachea 0.11 per cent; esophagus 0.07 per cent; aorta 0.14 per cent; submaxillary gland 0.06 per cent; costal cartilage 0.02 per cent.

*** Additional tissues: testicle 0.25 per cent; thyroid 0.20 per cent; salivary gland 0.37 per cent; bladder 0.16 per cent. Bladder urine at death contained no sugar.

† Additional tissues: pancreas remnant 0.26 per cent; thyroid, trace; esophagus 0.25 per cent; trachea 0.26 per cent; uterus 0.41 per cent; submaxillary gland 0.11 per cent; bladder 0.29 per cent; aorta 0.38 per cent.

‡ Muscle taken before sugar was given showed 0.17 per cent, after hydrolysis 0.18 per cent. Only a trace of glycogen was present. Muscle after sugar injection contained 0.08 per cent glycogen and liver 0.06 per cent. Plasma sugar directly after injection of sugar was 1.78 per cent.

TABLE X.

Comparison between Normal and Diabetic Animals. Liver and Muscle Tissues.

Plasma dextrose.		Liver dextrose.		Muscle dextrose.		Heart muscle dextrose.	
Normal.	Diabetic.	Normal.	Diabetic.	Normal.	Diabetic.	Normal.	Diabetic.
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0.21	0.23	0.80	0.59	0.06	0.09	0.05	0.06
0.24		0.37		0.11		0.12	
0.25				0.09		0.29	
0.25		0.56		0.27		0.31	
0.26	0.26	0.23	0.26	0.13	0.06	0.21	0.04
0.27	0.27	0.32	0.83	0.10	0.05	0.22	0.15
0.29	0.29	0.51	0.40	0.14	0.07	0.23	0.14
0.32	0.29	0.29	0.22	0.25	0.05	0.21	0.11
0.40	0.38	0.62	0.59	0.10	0.21	0.08	
0.41	0.44	0.55	0.20	0.25	0.02	0.22	0.08
	0.53		0.34		0.05		0.18
	0.56		0.37		0.26		0.28
	0.58		0.49		0.12		0.13
0.63	0.68	0.47	0.65	0.21	0.13	0.08	0.33
0.83	0.71	0.75	0.35	0.33	0.14	0.33	0.10
1.05	1.00	0.75	0.48	0.41	0.33	0.56	0.27
	1.43		0.95		0.25		0.45

Increase in the Reducing Power of the Tissue Extracts after Boiling with Hydrochloric Acid.

In many of the experiments the tissue extracts were hydrolyzed to determine the amount of sugar present as polysaccharides. The results are of sufficient interest to make it desirable to arrange these data separately in Table XI.

It is noted that occasionally, but not always, there is an appreciable increase in the reducing power of the muscle extracts after hydrolysis. This applies both to skeletal and heart muscles, although the percentage increase in the thigh muscle is often greater than in the heart. In all the normal dogs, only three, Nos. 355, 361, and P III, failed to show an appreciable increase in the dextrose values on boiling with hydrochloric acid. The reducing power of the muscle extract in Dog P II is nearly trebled on hydrolysis. The diabetic dogs are equally divided, Dogs 322

TABLE XI.

Increase in the Reducing Power of the Tissue Extracts after Hydrolysis.

No. of experiment.	Plasma sugar.	Liver.		Skeletal muscle.		Heart.		Stomach.	
		Before hydrolysis.	After hydrolysis.	Before hydrolysis.	After hydrolysis.	Before hydrolysis.	After hydrolysis.	Before hydrolysis.	After hydrolysis.
				Normal.					
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
N IV	0.11	0.26	0.27	0.07	0.10	0.20	0.25	0.04	0.04
350	0.24	0.37	0.37	0.11	0.20				
347	0.19	0.86	0.85	0.11	0.16	0.13	0.17		
I II	0.25	0.56	0.58	0.27	0.35	0.31	0.35	0.13	0.16
355	0.83	0.75	0.72	0.33	0.32	0.33	0.36	0.46	0.46
361	0.32	0.29	0.27	0.25	0.24	0.21	0.20	0.11	0.12
A II	0.36	0.52	0.52	0.21	0.36	0.23	0.25	0.14	0.17
A III	0.29	0.51	0.56	0.14	0.28	0.23	0.27	0.15	0.17
353	0.18			0.08	0.12				
P II	0.26	0.23	0.27	0.13	0.32	0.21	0.29	0.15	0.19
P III	0.47	0.55	0.57	0.25	0.29	0.26	0.27	0.22	0.27
				Diabetic.					
322	0.56	0.26	0.26	0.28	0.29			0.29	0.29
397	0.38	0.59	0.59	0.21	0.30				
271	1.00	0.48	0.48	0.33	0.32	0.27	0.29		
D I	1.43	0.95	0.95	0.25	0.47	0.45	0.57	0.56	0.53

and 271 show no increase, No. 397 shows a slight increase, and No. D I nearly doubles the dextrose on hydrolysis. Apparently the presence or absence of hydrolyzable sugar in the muscles does not depend on the presence or absence of diabetes. Although the administration of sugar both in normal and diabetic animals increases the amount of dextrose found after hydrolysis, when this amount is compared with the sugar present as dextrose the percentage increase is about the same with or without sugar administration.

Examination of Table XI reveals further that the striated muscle of dogs is the only tissue which contains any appreciable amount of sugar in the polysaccharide state. Extracts of stomach and liver, as well as kidney, lung, and skin, never show a change in reducing power on hydrolysis that is not well within the limits of error in experimental technique.

Amount of Dextrose Recovered after Injection.

In several of our experiments the data at hand were utilized to estimate what proportion of dextrose injected into the animal could be accounted for (Table XII).

TABLE XII.

Amount of Dextrose Recovered after Injection.

No. of experiment.	Weight of dog.	Route of sugar administration.	Sugar given.	Sugar recovered.		Time after sugar given before analysis.	Remarks.
	kg.		*gm.*	*gm.*	*per cent*	*hrs.*	
350*	15.0	By mouth.	97	94.4	97	1	
P III	15.5	Intravenous.	62	57.2	92	1½	
347**	16.0	By mouth.	192	159.0	83	2	
D I	18.7	Intravenous.	76	53.2	70	1½	
297	16.3	"	24	15.4	64	½	Partial pancreatectomy but not glycosuric.
I_2	14.5	"	52	31.9	61	1½	
P II	11.0	"	40	21.8	55	1½	

* Experiment was performed under conditions similar to those in Lusk's metabolism work (19).

** Levulose was the sugar used in this experiment.

As it was not possible to estimate the respiratory metabolism, Dog 350 was given sugar under conditions similar to those of Lusk (19) when the amount of sugar burned was followed for several hours after its introduction into the stomach. A 15 kilo dog was given 97 gm. of dextrose in 170 cc. of water by stomach tube and killed an hour later. In estimating the sugar in the tissues at the beginning of the experiments the average of the normal values in Table I was used. In computing the amount of sugar recovered the volume of blood was estimated as amounting to one-twelfth of the body weight (20); the muscles as three-sevenths of the body weight. Details of the calculation are given below. The weight of the dog was 15 kilos.

	gm.	
1. Amount recovered from stomach contents	77.5	
2. " " " " itself	5.1	
3. " " " intestines	0.9	
4. " " " blood. 1250 × 0.10 per cent	1.3	
5. Amount recovered in muscles. 6429 × 0.05 per cent	3.2	
6. Amount recovered in liver, kidneys, lungs, and skin	1.9	
7. Amount burned computed on basis of Lusk's experiment	4.5	
Total recovered	94.4	
Amount given	97.0	
Amount unaccounted for	2.6	
Recovered	97	per cent.

This method for estimating the recovered sugar is used in the other animals reported except that no estimation of amounts that may have been burned was possible. Following levulose by mouth and dextrose intravenously the amounts of sugar burned are unknown. Glycogen estimations were not made in all cases. Dogs D I and P II showed no glycogen, while in Dog P III there was 0.16 per cent in the liver and 0.34 per cent in the muscles. Variable amounts of sugar were accounted for. In Experiment 350, where it was possible to estimate the dextrose burned, 97 per cent was recovered. In Dog P III after intravenous dextrose we were able to account for 92 per cent. The amount of dextrose burned in each case is important in attempting to account for the disappearance of dextrose after injection, and especially so when sugar is given intravenously, because Lusk has shown that the higher the concentration of metabolisable material in the blood stream, the higher the metabolism.

Only one opportunity to examine human tissue presented itself during this investigation. A boy 9 years old died in diabetic coma, 2 years after onset. 24 hours before death his blood sugar was 0.50 per cent, and his liver, obtained 1 hour after death, contained 0.55 per cent of dextrose; no change after hydrolysis. There was no glycogen in the liver.

DISCUSSION.

From the data presented it is clear that under widely varying conditions the amount of carbohydrate existing as dextrose in the tissues at any one time is small. The tissue of most importance is muscle, for it comprises three-sevenths of the body weight and by its mass could be responsible for storing large amounts of free dextrose. Four normal animals, having an average blood sugar of 0.11 per cent, had an average of 0.08 per cent in the skeletal and heart muscle. These values are much lower than the 0.2 to 0.4 per cent found by Pavy, who does not give any blood sugar figures. Although sugar administration increases the amounts of sugar to be found in the tissues, its concentration is always low and not commensurate with the increase in blood sugar. The highest value, 0.41 per cent, for muscle sugar was found after intravenous injection of 5.5 gm. per kilo, in which the plasma sugar rose to 1.05 per cent. Except in the phlorhizinized dog, No. 353, with a plasma sugar of 0.08 per cent and a muscle sugar of 0.08 per cent, we have never found dextrose in the muscle to equal that in the blood. Frequently the muscle sugar was only half as concentrated as the blood sugar, and in some instances in normal animals, as Dog 337, amounted to only one-fourth.

Diabetic animals present little difference in respect to dextrose in muscles. The amounts found are small, and apparently not influenced by the type of diabetes or sugar administration. As in the normal animals, the controlling factor seems to be the concentration of dextrose in the blood. The point of difference between the normal and diabetic animal is that when comparative blood sugar values are taken the muscle of the diabetic animals contains less dextrose than the normal. This fact suggests that diabetic tissues are more impermeable to sugar than normal ones, and coincides with the results of previous workers, that sugar injected into the veins of diabetic animals does not leave the blood so rapidly as it does in normal animals. Just what relation this phenomenon may bear to glycogenesis and the oxidation of glucose in the organism is unknown and difficult to conjecture. As all the tissues contained more or less blood, the actual amount of dextrose in the muscle substance must have been very small indeed. It is reasonable to expect that the amounts of blood remaining in the

tissues in animals killed under similar conditions were fairly constant. Therefore the difference between normal and diabetic animals gives support to the belief that, contrary to the findings of Albertoni and Monetti, dextrose does exist in the muscles, at least in normal animals.

That body tissues ordinarily contain only small amounts of free dextrose has an important bearing on all discussion of the fate and probable disposition of dextrose in the organism. Body fluids and tissues have been credited with the same concentration of dextrose as exists in the blood. In amount at least, this allowance is far too liberal. We have no way of telling now nearly the animal experiments correspond to what takes place in normal and diabetic men. But granting that the fate of glucose is somewhat similar, it is no longer possible to explain the retention of carbohydrate by diabetics as in the form of dextrose distributed throughout the tissue and body fluids. In the case of Gerald S. reported by Allen and Du Bois (21), the non-protein respiratory metabolism showed over a period of 2 weeks 438 calories derived from carbohydrates, while the carbohydrates in the food were only 2 gm. daily. As the individual weighed 40 kilos, the muscle tissue computed at three-sevenths of the body weight amounts to 17.7 kilos. Allowing a diminution of 0.10 per cent in the concentration of dextrose in the muscles during this time as contributing to the carbohydrate calories, only 17.7 gm. are available. An allowance of 0.10 per cent reduction in the muscles, judging from the results of animal experimentation, is liberal when one considers that Gerald S.'s blood sugar varied between 0.156 per cent and 0.208 per cent during the period of observation in question. As Allen and Du Bois and others have shown that acetone bodies cannot exist in quantities sufficient to account for more than a small part of the increase in the respiratory quotient of these cases, the evidence points strongly to glycogen as furnishing the extra calories.

It is necessary in this connection to consider the accuracy with which it is possible to estimate the amount of carbohydrate burned over long periods when computing the amount of sugar retained by the diabetic individual. Under the usual conditions there is a possible error of 6 to 10 per cent in the determination of the respiratory quotient because the second figure in the quotient is

accurate to within 2 or 3 only, although under ideal circumstances an error of only 1 in the second place may be secured. Hence a man could burn 50 gm. of dextrose at the rate of 2 gm. per hour in 24 hours, impossible of detection by the calorimeter. On the other hand, an organism may be accredited with the combustion of more sugar than actually occurs, at least within these limits. In diabetes the situation is further complicated when acidosis is present.

With the exception of the liver, which holds a particular place in carbohydrate metabolism, the several tissues examined besides muscle reveal little that we consider of importance. No evidence has come to light that any of the tissues have unusual power to store dextrose in high concentration. In general it may be said of the minor tissues that the concentration of dextrose varies more or less with vascularity. The brain, which contains very little blood, contains exceptionally low amounts of dextrose. In Dog 352 after intravenous dextrose only 0.11 per cent was found in the brain. The average under the varying conditions is considerably below this figure.

Analyses were made of the stomach for the purpose of comparing smooth with striated muscle. In nearly every instance the concentration in the stomach musculature approximated that found in the striated muscles.

The determination of dextrose in the liver involves several difficulties not easy to overcome. From examination of Table III it is evident that the manner in which the animal is killed, as well as the rapidity with which enzyme action is checked, is most important. But although these factors were controlled as well as possible, there exists a wide variation in the amount of dextrose found. This variation seems most probably due to difference in glycogenolysis in the several animals. In the normal animals, P II and P III, known to be glycogen-free, and in the diabetic dogs, the liver dextrose was relatively low. Only two of the diabetic dogs, Nos. 300 and 292, have concentrations of dextrose in the liver higher than that in the plasma. In Experiment 300, owing to an accident, the liver was not thrust into boiling water for 3 or 4 minutes after excision. Dog 292 was killed early in the work by bleeding, before the effect of bleeding on glycogenolysis in the liver was appreciated. In the light of Bang's experi-

ments the higher values in the livers of normal animals might be interpreted as meaning an ability, not possessed by the diabetic dogs, of storing dextrose in higher concentration than it existed in the blood. By taking especial precautions to prevent glycogenolysis we were unable to confirm Bang's results, nor are we inclined to attribute the difference in values between normal and diabetic animals as having any such significance. The marked rapidity with which glycogenolysis occurs in the liver, as compared to the muscles, has been observed by Panormoff (22) and others.

By hydrolysis with hydrochloric acid the extracts of heart and skeletal muscle showed an increase in their power to reduce copper. No attempt was made to determine the nature of the substance yielding extra sugar, but previous work suggests that it may be due largely to a biose, probably maltose.

By treating the extract of dog muscle with phenylhydrazine hydrochloride Panormoff (22) obtained a crystalline substance with a low melting point which he was unable to identify. An osazone obtained from the liver isolated by Röhmann (23) was considered by them to be isomaltose, but no details are reported. Pavy and Siau (24) by first precipitating the proteins of the blood with alcohol and extracting the coagulum with water, obtained an extract which yielded a copper-reducing substance after boiling with hydrochloric acid. An osazone was obtained melting at 153° and considered by them to be isomaltosazone because of its resemblance to Fischer's substance which he called "isomaltose." A similar osazone was isolated by Osborne and Zobel (25) melting at 153°, but on further purification they were able to raise the melting point from 153° to 162°. Hence they concluded that the substance was not isomaltosazone, but in reality maltosazone contaminated by dextrinous bodies. Furthermore, these workers showed that when muscles were washed free of blood by salt solution the muscle juices hydrolyzed glycogen into a substance which they considered to be maltose. The results of Levene and Meyer (26) are interesting in this connection. They found that if muscle juice (rabbits) and pancreas extract were allowed to act on a solution of dextrose a condensation occurred. A biosazone was isolated with a melting point of 200°.

The evidence at hand strongly favors the presence in muscle of higher sugars or polysaccharides, of which maltose probably constitutes the larger part. The fact that no hydrolyzable sugar exists in other tissues than muscle is interesting, but permits of no special interpretation, particularly because it occurs in both normal and diabetic animals.

Attempts to account for all the dextrose injected into the ani-

mal, similar to the experiences of other investigators, have not been wholly satisfactory. The difficulties attending such an investigation have been discussed by Joslin (27). After injection of dextrose the amount excreted unutilized in the urine and the quantity burned may be determined with some accuracy, provided a calorimeter is available. The amount retained as dextrose in the blood and tissues is much more difficult to estimate, as is also the amount transformed into glycogen, polysaccharides, and fat. Little is known about the incompletely oxidized products of sugar. Attention should be called to the frequently neglected changes in blood volume following the intravenous injection of large amounts of dextrose. A calorimeter was not available in our work; hence our observations cannot be considered complete. To overcome this difficulty partially Dog 350 was given dextrose under conditions similar to those used by Lusk in determining the amount and rate at which dextrose was oxidized after mouth administration. We were able to account for 97 per cent of the administered dextrose in this dog. Where levulose, 12 gm. per kilo, was introduced by stomach tube in Dog 347, only 83 per cent was accounted for, but this did not include any estimate of the amount burned. Johansson (28) found that the CO_2 output after levulose administration in animals and men was frequently double what it was after the injection of dextrose. Considerable levulose may have been oxidized in the 2 hours elapsing between its injection and the killing of the animal. After the intravenous administration of 4 gm. per kilo and killing in $1\frac{1}{2}$ hours, 92 per cent of the amount given could be accounted for, while in others less amounts were recovered. During the short period between sugar injection and the killing of the animal it is usually taken for granted that little glycogen is formed. Freund and Popper's (29) work confirms this view. But that glycogen may be formed in a short period of time is shown in Dog P III, which was made glycogen-free by phlorhizin and adrenalin (the muscles proved to be free of glycogen by analysis before sugar was given), and which had an appreciable amount of glycogen, 0.34 per cent, in the muscles $1\frac{1}{2}$ hours after dextrose administration. It is possible, however, that glycogen was unevenly distributed in the muscles and the sample taken at the beginning of the experiment did not have any, but the remaining muscle still contained small amounts.

The conditions governing the formation of glycogen are not well known. Johansson observed, in men who had their glycogen reserve depleted by starvation or severe muscular work, that the CO_2 output after sugar administration was much less than in men with a normal amount of glycogen. He logically interpreted this fact as indicating a much more rapid formation of glycogen following ingestion of sugar by glycogen-poor organisms than under normal conditions. Other not well known factors in glycogen formation doubtless play a part. When all the difficulties encountered in attempting to trace the fate of ingested dextrose are considered, it is not strange that wide variations in results are found, also that there is more or less sugar for which no account can be given.

SUMMARY.

1. The concentration of dextrose has been determined in muscle, liver, heart, kidney, spleen, pancreas, stomach, intestine, skin, lung, and eye tissues of normal and diabetic animals, under varying conditions. In a few instances the dextrose has been estimated in the bladder, parotid glands, esophagus, uterus, tongue, diaphragm, thyroid, aorta, trachea, and brain.

2. No difference in the amount of dextrose to be found in either normal or diabetic tissues is caused by variation in the manner of producing a hyperglycemia; that is, administration by mouth, subcutaneously, intravenously, or intraperitoneally.

3. The concentration of dextrose in the tissues varies directly with the degree of hyperglycemia. The largest amount of sugar was found in the liver, the smallest quantity in the brain, the difference being largely accounted for by difference in vascularity.

4. The concentration of dextrose in the tissues is invariably lower than the blood sugar except in the liver, where the higher concentrations found may be explained by rapid glycogenolysis. In the muscles the amount of dextrose is low, varying in normal animals between 0.04 per cent when the blood sugar is 0.10 per cent and 0.41 per cent when the blood sugar reaches 1.05 per cent after intravenous sugar; while in diabetic animals the muscle sugar falls within the normal limits even though the hyperglycemia rises as high as 1.43 per cent.

5. Normal animals have a higher concentration of dextrose in

striated muscle than diabetic animals when the levels of blood sugar are comparable.

6. Bleeding of the animal causes a rapid glycogenolysis in the liver.

7. Adrenalin produces no change in tissue sugars different from that found when hyperglycemia is caused by other means.

8. In the striated muscles of many but not all normal and diabetic animals there is an increase in the reducing power of the extract after boiling with hydrochloric acid. No such changes could be demonstrated in the extracts of other tissues. The polysaccharide mother substance of the sugar formed by hydrolysis is not glycogen, and may be present when glycogen is not.

9. Amounts of dextrose varying between 55 and 97 per cent of the amount injected were accounted for. The maximum of 97 per cent was recovered in an animal where it was possible to calculate the amount of sugar burned.

10. The liver of a boy who died in diabetic coma, obtained 1 hour after death, contained 0.55 per cent of dextrose and no glycogen. His blood sugar 24 hours before death was 0.50 per cent.

BIBLIOGRAPHY.

1. Bernard, C., Nouvelle fonction du foie, considéré comme organe producteur de malière sucrée chez l'homme et les animaux, Paris, 1853.
2. Dalton, J. C., A Treatise of Human Physiology, Philadelphia, 1871, 5th edition.
3. Von Mering, *Arch. Physiol.*, 1877, 379.
4. Seegen, J., und Kratschmer, F., *Arch. ges. Physiol.*, 1880, xxii, 214.
5. Girard, H., *Arch. ges. Physiol.*, 1887, xli, 294.
6. Pavy, F. W., The Physiology of the Carbohydrates, London, 1894.
7. Butte, L., *Compt. rend. Soc. biol.*, 1896, iii, 274.
8. Bang, I., Der Blutzucker, Wiesbaden, 1913.
9. Hédon, E., *Arch. physiol. norm. et path.*, 1892, xxiv, 245.
10. Levene, P. A., *J. Physiol.*, 1894–95, xvii, 259.
11. Embden, G., *Beitr. chem. Phys. u. Path.*, 1905, vi, 44.
12. Albertoni, P., and Monetti, G., *Arch. ital. biol.*, 1915, lxiv, 1, abstracted in *Physiol. Abstr.*, 1916, i, 287.
13. Kleiner, I. S., *J. Exp. Med.*, 1916, xxiii, 507.
14. Griesinger, W., *Arch. physiol. Heilk.*, 1859, iii, 1.
15. Benedict, S. R., *J. Biol. Chem.*, 1911, ix, 57.
16. Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.
17. Allen, F. M., Glycosuria and Diabetes, Cambridge, 1913.
18. Sansum, W. D., and Woodyatt, R. T., *J. Biol. Chem.*, 1915, xxi, 1.

19. Lusk, G., *J. Biol. Chem.*, 1912–13, xiii, 27.
20. Keith, N. M., Rowntree, L. G., and Geraghty, J. T., *Arch. Int. Med.*, 1915, xvi, 547.
21. Allen, F. M., and Du Bois, E. F., *Arch. Int. Med.*, 1916, xvii, 1010.
22. Panormoff, A., *Z. physiol. Chem.*, 1893, xvii, 596.
23. Röhmann, F., *Centralbl. med. Wissensch.*, 1893, xxxi, 849.
24. Pavy, F. W., and Siau, R. L., *J. Physiol.*, 1900–01, xxvi, 282.
25. Osborne, W. A., and Zobel, S., *J. Physiol.*, 1903, xxix, 1.
26. Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1911, ix, 97.
27. Joslin, E. P., *Arch. Int. Med.*, 1915, xvi, 694.
28. Johansson, J. E., *Skand. Arch. Physiol.*, 1909, xxi, 1.
29. Freund, E., and Popper, H., *Biochem. Z.*, 1912, xli, 56.
30. Michaelis, L., and Rona, P., *Biochem. Z.*, 1908, vii, 329.

THE EFFECT OF HIGH TEMPERATURES ON THE NUTRITIVE VALUE OF FOODS.

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In an earlier publication[1] from this laboratory results were cited which indicated that young rats are unable to grow on a diet that has been subjected to high temperatures. Many investigators believe that the so called accessories are destroyed by exposure to heat, and as that is a point of great importance, further studies were made on the nutritive properties of heated foods.

In these investigations white rats were used as experimental animals. Many of them were at least half grown when the feeding trials began, but some were quite immature. In some cases corn constituted the ration, in others a mixture of corn and egg white, one or both of which had been heated in an autoclave. In every case a salt mixture was added to the diet to compensate for the mineral deficiencies of maize. The heating process was continued for 6 hours at 30 pounds' pressure.

It soon became evident that heating the egg white had little or no effect; but in every case in which the corn was heated, the diet was inadequate in some respect, and the animals were unable to maintain their body weights. These facts are presented in greater detail in Charts 1 to 4.

It has been suggested, however, that some factor other than that of accessories was altered. If such were the case probability seemed to indicate that the protein of the ration had been changed in some way. In order to investigate that point, a comparatively pure protein, egg white, was heated in an autoclave for 6 hours at 30 pounds' pressure. This was carefully dried, and combined

[1] **Hogan**, A. G., *J. Biol. Chem.*, 1916, xxvii, 193.

with protein-free milk, butter, starch, and agar, in such proportions that the protein formed approximately 9 per cent of the ration. This diet did not permit maximum growth and possibly these rats grew somewhat more slowly than other animals receiving a similar ration in which the protein had not been heated. Evidently, however, the nutritive value of the protein had not been seriously impaired.

A series of feeding trials similar to the one just described was conducted with casein in the ration instead of egg white. In these experiments the casein was heated in an autoclave for 2 hours, but at different pressures. In one case the protein was heated at 15 pounds', in another at 30 pounds', and in a third at 45 pounds' pressure. A control lot received unheated casein. In this series the protein formed approximately 12 per cent of the ration. In all cases animals of the same sex grew at approximately the same rate, indicating that the nutritive value of the casein had not been materially lowered by the high temperature.

The data are presented graphically in Charts 5 to 9.

SUMMARY.

The heating process described in this paper does not materially lower the nutritional value of protein.

It is suggested that one or more of the so called food "accessories" may be injured by high temperatures.

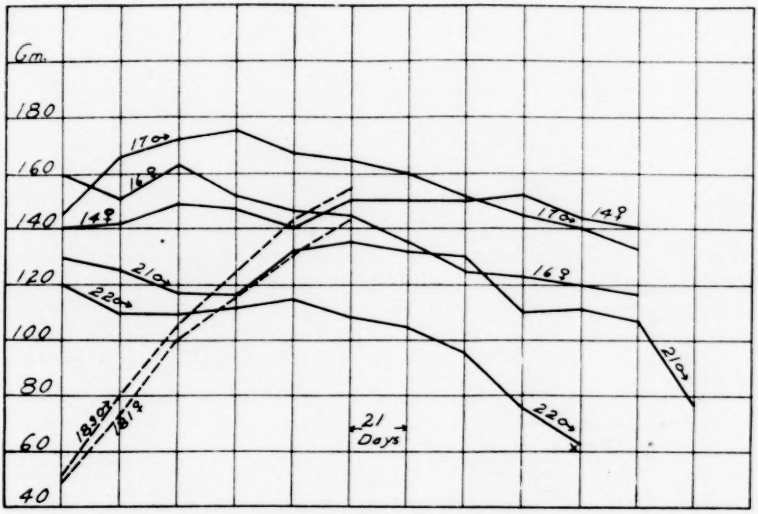

CHART 1. The animals shown in this chart received as their sole diet corn that had been heated in an autoclave. All of them slowly lost weight, and Rat 22 died. To show that rats are able to grow on corn that has not been heated, some additional curves are reproduced here. The broken lines representing Rats 181 and 183 are typical of a fairly large number that have grown practically to maturity in this laboratory on corn alone (that had not been heated). *x* at the end of a curve indicates the animal's death.

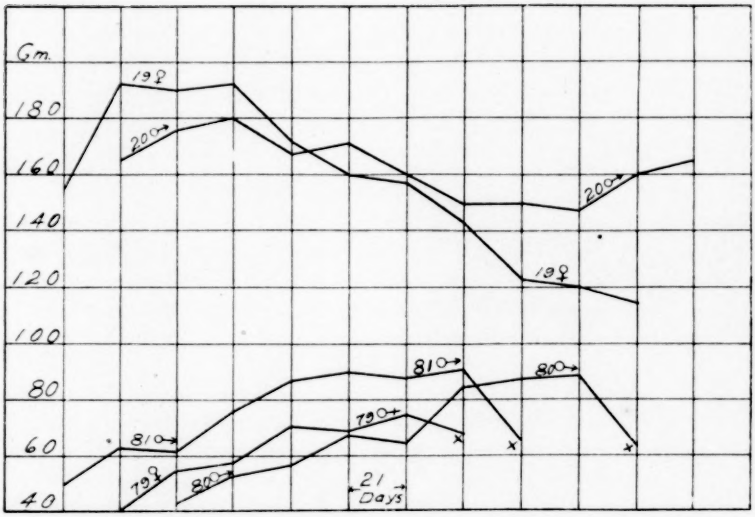

CHART 2. The animals shown in this chart received a diet similar to the one fed to the rats in Chart 1. This ration contained a much larger percentage of protein, however. Enough of the autoclaved egg white was added to make the protein content of the diet approximately 20 per cent. The younger animals failed to grow and finally died. During the experimental period the older animals steadily lost weight. *x* at the end of a curve indicates the animal's death.

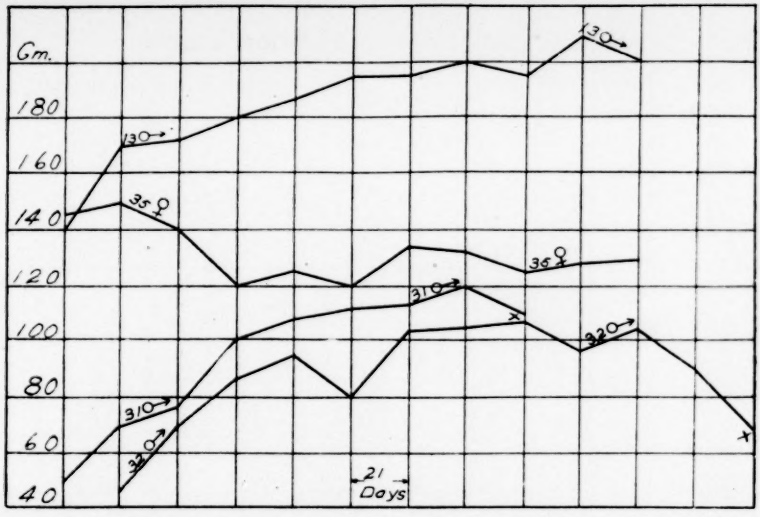

CHART 3. These animals received corn that had been subjected to high temperatures. In addition they received unheated egg white, in amounts comparable to that given to the rats shown in Chart 2. The two younger animals grew slowly for a time, but finally died. Of the two older rats, the male made some growth, but the female gradually lost weight. *x* at the end of a curve indicates the animal's death.

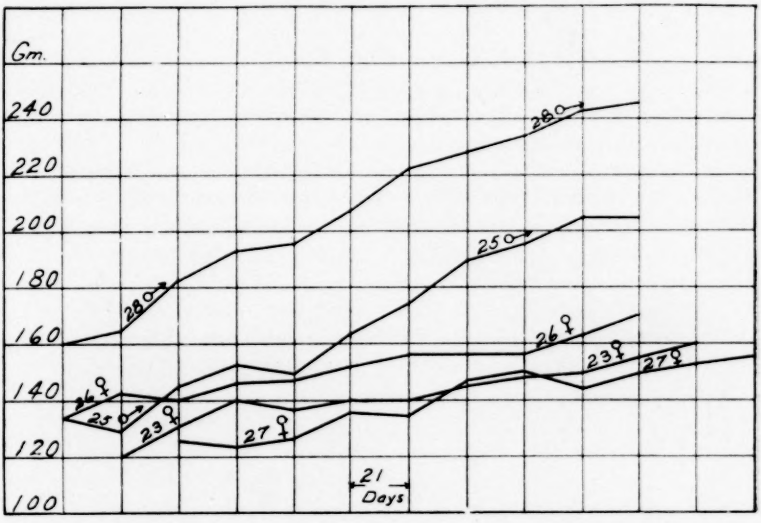

CHART 4. The diet received by these animals consisted of unheated corn and autoclaved egg white. All animals grew slowly, but at the usual rate for this ration.

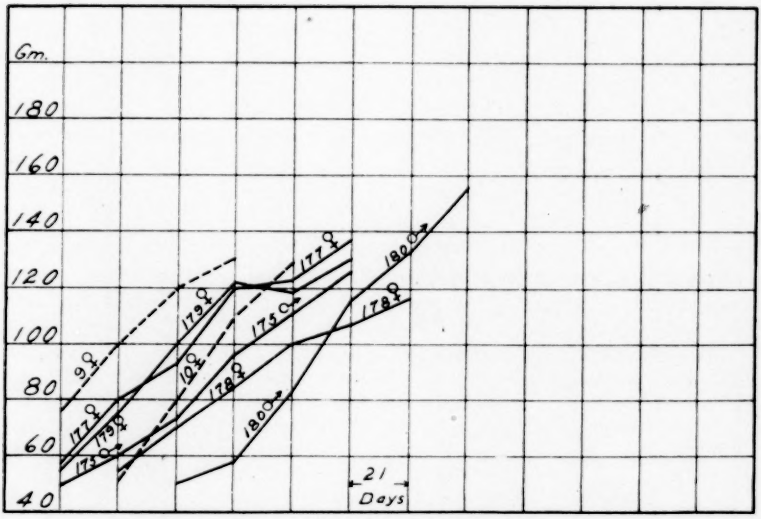

CHART 5. The protein constituted 9 per cent of the ration in this case, and consisted of egg white instead of casein. The egg white had been heated in the autoclave for 6 hours at 30 pounds' pressure. The broken lines represent the growth of rats on a similar diet, in which the egg white had not been heated. The composition of the ration was as follows.

	gm.
Egg white	100
Protein-free milk	257
Butter	300
Starch	325
Agar	20

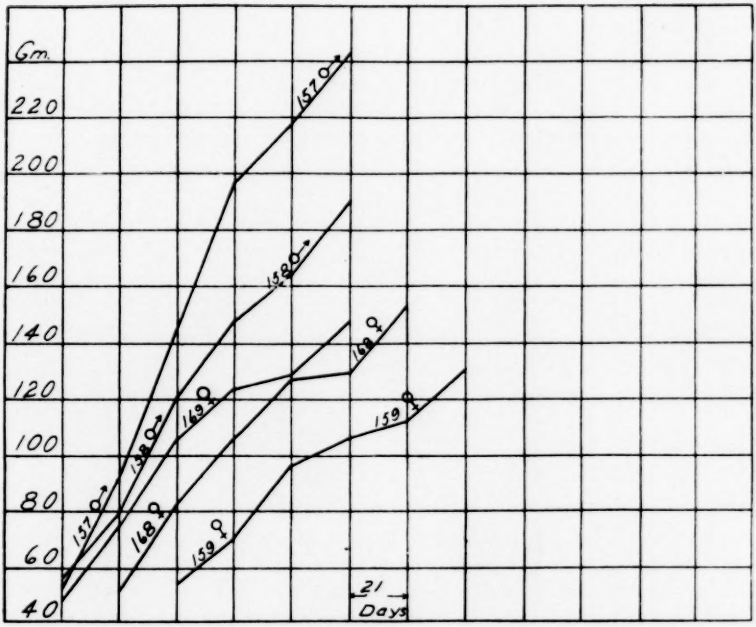

CHART 6. The diet fed to the animals shown above included approximately 12 per cent of unheated casein. These rats serve as controls for Charts 7, 8, and 9. Of the males, Rat 157, and of the females, Rat 169 grew most rapidly, and for convenience their growth curves are inserted in the following charts. The ration is as follows.

	gm.
Casein	152
Protein-free milk	257
Butter	300
Starch	295
Agar	20

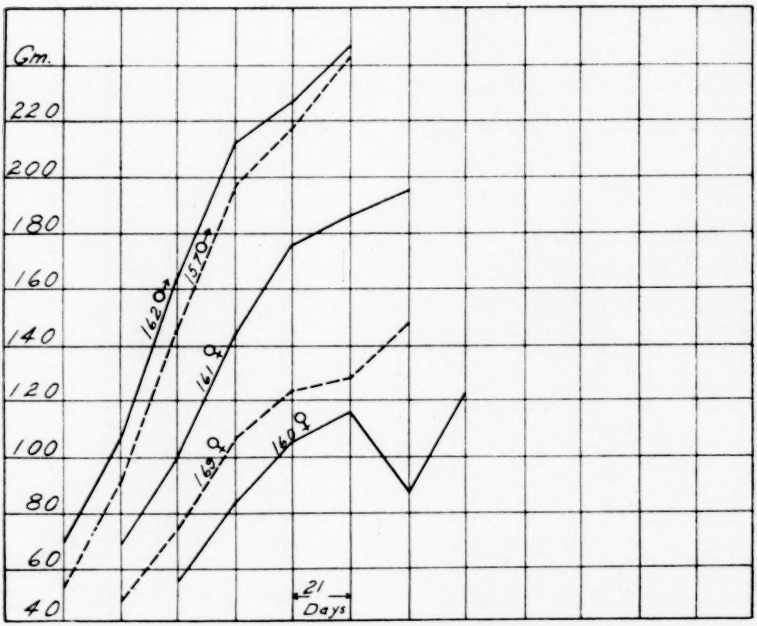

CHART 7. The protein in the ration of these animals had been heated in an autoclave for 2 hours at 15 pounds' pressure. The broken lines show the growth of control animals, taken from Chart 6.

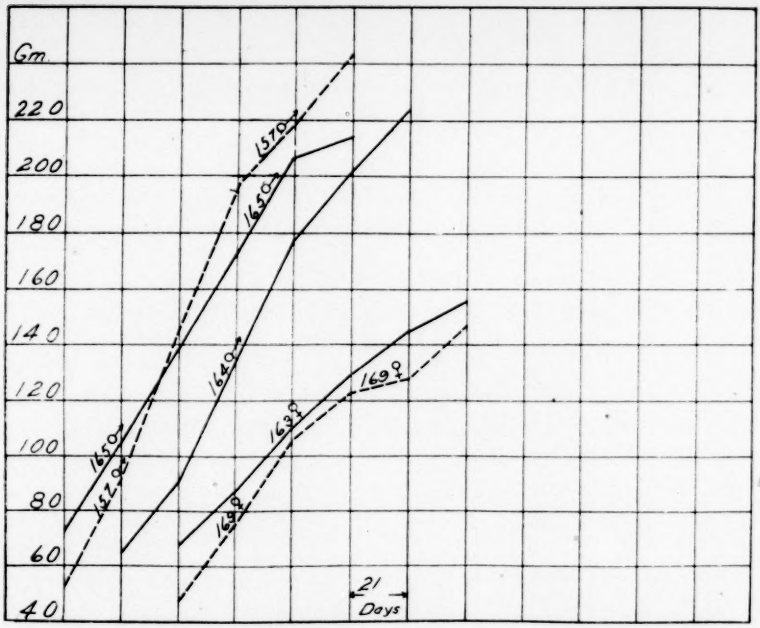

CHART 8. The casein used in preparing the diets of these animals had been heated at 30 pounds' pressure for 2 hours. Evidently the nutritional value of the casein had not been impaired.

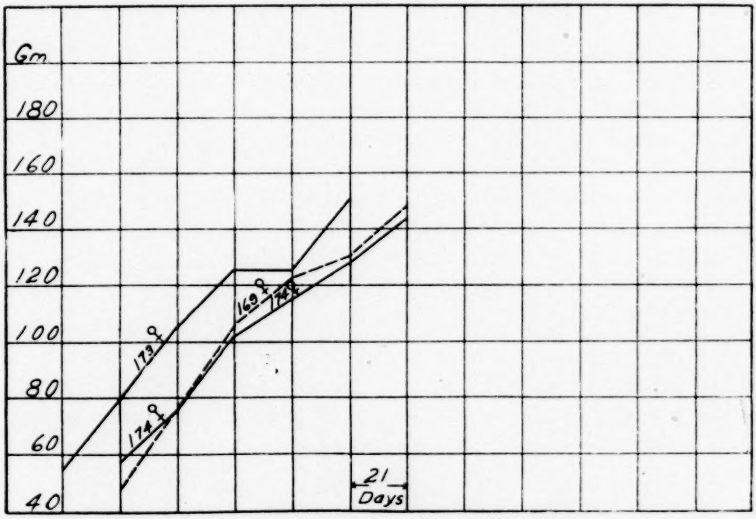

CHART 9. These animals received casein that had been heated in the autoclave for 2 hours at 45 pounds' pressure. As both of these animals were females, the curve of Rat 157, Chart 6, is omitted.

A QUANTITATIVE TEST FOR SMALL AMOUNTS OF SUGAR IN THE URINE.

By ALMA HILLER.

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(Received for publication, March 6, 1917.)

A rapid, accurate method for the determination of small amounts of sugar in the urine is not available at present. To overcome this uncertainty, a micro method was adopted. By adapting the blood sugar method of Lewis and Benedict,[1] even traces of urinary sugar may be determined quantitatively with great precision.

Since the colorimetric estimation of Lewis and Benedict depends upon the reaction of the sugar with alkali and picric acid, and a similar color reaction is obtained by the action of alkali and picric acid on the creatinine and other substances normally present in urine, these must be removed before the method can be applied. Normal urines without sugar yield a color reaction with the Lewis and Benedict method equivalent to 0.05 to 0.1 per cent glucose. In order to remove these interfering substances, the process recently suggested by Folin[2] was used.

The complete method is as follows:

To about 10 cc. of urine are added 2 gm. of picric acid and 2 gm. of bone-black, the mixture is shaken for about 5 minutes and filtered.[2] 2 cc. of this filtrate are used for the determination, and placed in a 25 cc. volumetric flask. Then 15 cc. of saturated aqueous solution of picric acid are added and the flask is filled to the mark with distilled water and shaken. Filtration at this point is not necessary. 8 cc. aliquots are measured into large Jena test-tubes for duplicate determinations. 2 cc. of saturated picric acid solution and exactly 1 cc. of 10 per cent sodium carbonate are added, then one drop of mineral oil, and the contents of the flask are evaporated rapidly over a direct flame until pre-

[1] Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

[2] Folin, O., *J. Biol. Chem.*, 1915, xxii, 327.

cipitation occurs. About 3 cc. of water are added, the tube is again heated to boiling to dissolve the precipitate, the contents of the tube are cooled, transferred quantitatively to a 10 cc. volumetric flask, made up to mark, shaken, and then filtered through cotton into the colorimeter chamber. The color is compared with a picramic acid standard:

Picramic acid	0.064 gm.
Sodium carbonate (anhydrous)	0.100 "
Water to make	1,000 cc.

Calculation:

$$\text{Per cent of sugar} = \frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 0.1$$

Tables I and II indicate that creatinine and other substances which give a color reaction with alkaline picrate are entirely removed by the picric acid and bone-black process.

TABLE I.

The Method Applied to Glucose Added to Urine.

Specimen.	Substance added.	Preliminary treatment.	Result, glucose.
			per cent
Urine.	None.	None.	0.084
"	0.1 per cent glucose.	"	0.183
"	0.1 " " "	Bone-black.	0.024
"	0.1 " " "	" + picric acid.	0.100
"	0.1 " " "	None.	0.128
"	0.1 " " "	Bone-black.	Not readable.
"	0.1 " " "	" + picric acid.	0.100

TABLE II.

The Method Applied to a Pentose.

Specimen.	Substance added.	Preliminary treatment.	Result, glucose.
			per cent
Urine.	None.	None.	0.054
"	0.1 per cent *l*-xylose.	"	0.153
"	0.1 " " "	Bone-black.	0.070
"	0.1 " " "	Picric acid.	0.153
"	0.1 " " "	Bone-black + picric acid.	0.101

As the tables show, this procedure is applicable to urines containing pentoses as well as glucose. The method proved to be valuable in estimating the quantity of sugar excreted in a case of pentosuria, in which the concentration of this sugar was only 0.1 to 0.3 per cent.

After the completion of the above article the writer's attention was called to a method published by V. C. Myers,[3] which is similar to, but not identical with her own.

[3] Myers, V. C., *Proc. Soc. Exp. Biol. and Med.*, 1916, xiii, 178.

THE IDENTIFICATION OF THE PENTOSE IN A CASE OF PENTOSURIA.

By ALMA HILLER.

(From the Medical Clinic of the Johns Hopkins Hospital, Baltimore.)

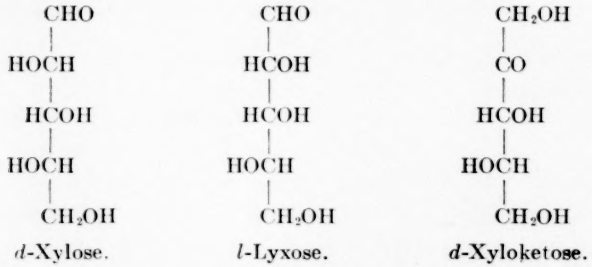
(Received for publication, March 6, 1917.)

Attempts to analyze the pentose eliminated in cases of pentosuria have led to a variety of conclusions. Since the first proof of the excretion of a pentose by Salkowski in 1892 (1), the majority of the earlier cases were identified as inactive *d,l*-arabinose, chiefly by Neuberg (2). Instances of active, dextrorotatory pentose were demonstrated by Blumenthal (3), Rosenfeld (4), and Schüler (5). Active levorotatory pentose was found by Adler (6) and Blum (7). Elliott and Raper (8) attempted to identify their pentose as *d,l*-ribose. Luzzatto (9) and Klercker (10) both demonstrated the dextrorotatory *l*-arabinose. More recently, Levene and La Forge (11) established the identity of their pentose with the osazone of *d*-xylose. Zerner and Waltuch (12) also place their three cases in the *d*-xylose group.

The subject from whom the material for this investigation has been derived was a Russian tailor, aged 28. A diagnosis was made of a low grade of chronic nephritis, moderate hypertension, duodenal ulcer, and pentosuria. The period in which the urines were collected for the determinations reported here began with the first admission of the patient to the hospital in March, 1916, and continued for 8 months, the patient cooperating willingly while in his own home.

The pentose reported in the present observation gave an osazone which, after having been recrystallized from 30 per cent alcohol, melted at 159°C. An osazone formed from *l*-xylose and recrystallized in the same way melted at 161°C. Equal amounts of these osazones were then dissolved in hot 95 per cent alcohol, mixed, and the osazone was crystallized out. These crystals melted at 208°C., and decomposed at 215°C., showing the for-

mation of a mixed *d,l*-xylose, a characteristic reaction of the xylosazones (13), which was used by Zerner and Waltuch (12) and by Levene and La Forge (11) in their identifications. The characteristic mutarotation of xylosazone (Table III) further strengthened this point, and excluded the possibility of ribose or arabinose, and limited the choice to one of three pentoses, whose osazones give these reactions characteristic of *d*-xylosazone. These are *d*-xylose, *l*-lyxose, and *d*-xyloketose, which have the following formulas:



d-Xylose. *l*-Lyxose. ***d*-Xyloketose.**

By a determination of the direction of rotation of the sugar itself, it would be possible to eliminate one or two of the three pentoses. Since the concentration of pentose in the urine was very slight, varying between 0.1 and 0.3 per cent, the urine had to be concentrated before a satisfactory rotation could be read. The urine without concentration, decolorized by animal charcoal, gave no rotation. This may be accounted for by the fact that through treatment with animal charcoal some of the sugar in solution is adsorbed, especially in dilute solutions, and also to the fact that there are substances normally in urine which give a levorotation. This was shown by Zerner and Waltuch (12) in connection with their work on the specific rotation of the sugar. This apparent inactivity of the decolorized non-concentrated urine might lead to the possibility of mistaking the sugar for an inactive form. For this reason the urine was concentrated according to the method of Zerner and Waltuch with the preliminary precaution, suggested by Bang (14), to prevent loss of sugar by treatment with charcoal. The method in full will be presented in the experimental part. The rotation of a 1.25 per cent solution thus obtained was unmistakably dextro.

This would exclude the possibility of *d*-xylose, which is levorotatory. *l*-Lyxose has been demonstrated by Van Ekenstein and Blanksma (15), and is dextrorotatory. *d,l*-Xyloketose has been demonstrated by Neuberg (16), but *d*-xyloketose is, so far, unknown. But assuming, as Zerner and Waltuch do, that by analogy with *d*-fructose and *d*-sorbose the osazone and the sugar may rotate in the same direction, and that *d*-xyloketose would be dextrorotatory if it were found, the possibility is limited to two sugars, *l*-lyxose and *d*-xyloketose.

Comparing the observed rotations with the calculated ones, no identification with *l*-lyxose can be made. If, however, the levorotation value of normal urines (Zerner and Waltuch) is taken into consideration, approximate values might be obtained which approach the calculated values for *l*-lyxose.

As this evidence is not very convincing, attempts were made to establish the possible identity of this sugar as *d*-xyloketose. This was done by testing for the keto group by oxidation of the concentrated urine with bromine. As the experiments show, there was a very slight loss of the sugar, as compared with the entire loss of the sugar in the aldo control. As a further proof of the presence of the keto group, the test of Pinoff and Gude (17) with ammonium molybdate was applied to the concentrated urine, and the characteristic color reaction was obtained. The resorcinol reaction of Seliwanoff was also positive.

EXPERIMENTAL.

Method for Concentrating the Urine.—To about 500 cc. of urine, freed from toluene by filtration, enough alcohol was added to make a 20 per cent alcoholic solution. This was done in order to prevent the disappearance of sugar on decolorization with charcoal. According to Bang's statement (14) and the present observations, little sugar is lost if these precautions are taken. According to the original method of Zerner and Waltuch, as much as 20 per cent of the sugar could not be recovered, whereas with Bang's method there was a loss of only about 3 per cent. The treatment with blood charcoal and subsequent procedure was identical with that of Zerner and Waltuch.

TABLE I.

Rotation Values of the Concentrated Urine in a 2 Dm. Tube.

Reduction values.	Rotation observed.	Rotation calculated for *l*-lyxose.
per cent		
1.79	+0.24°	+0.49°
1.25	+0.21°	+0.34°

TABLE II.

Rotation of Urine Treated with d-Lyxose and Concentrated According to Zerner and Waltuch.

Reduction value.	Rotation observed.	Rotation calculated
per cent		
1.80	−0.70°	−0.50°

TABLE III.

Mutarotation of the Osazone, 0.025 Gm. in 1.25 Cc. of Pyridine Alcohol, Rotated in a 0.5 Dm. Tube.

Time after preparation of solution.	Rotation.
10 min.	+0.10°
25 "	+0.15°
4 hrs.	+0.24°
7 "	+0.40°

Methods for Identification and Separation of Aldo and Keto Sugars in Solution.

Two procedures were employed, a macro and a micro method, which in detail were as follows.

Macro Method.—The urine was concentrated in the above mentioned manner; in fact the same solution was used after its rotation had been observed. The quantity of sugar present was determined by the Fehling-Benedict method; the solution was then treated with liquid bromine, by adding twice the weight in bromine of the sugar found present. This solution was tightly stoppered and allowed to stand 48 hours, with occasional shaking. At the end of this interval any excess bromine was removed with a separating funnel, and the solution was freed of bromine by

distillation in vacuum at 50° until colorless. The solution was neutralized and another Fehling-Benedict determination made. As Tables IV and V show, in the case of a ketose there is very little loss on reduction. In the case of an aldose, the loss is 100 per cent.

TABLE IV.

Results of Brominization Method.

Solution.	Sugar present.		Loss.	
	Before brominization.	After brominization.		
	gm.	*gm.*	*gm.*	*per cent*
Urine concentrated	1.25	1.20	0.05	4
Levulose (ketose)	1.62	1.56	0.06	3.7
Glucose (aldose)	1.38	0.00	1.38	100

Micro Method.—In order to obviate the long process of decolorizing and evaporating under diminished pressure, a method was devised for making the determinations directly on the urine. Since the amounts of the sugar found in the urine were so small (usually 0.1 to 0.3 per cent), the blood sugar method of Lewis and Benedict was modified in order to determine the pentose in the urine quantitatively (18). This having been accomplished, the brominization was carried out as in the macro method.

TABLE V.

Results of Direct Brominization of Urine in the Present Case of Pentosuria.

Sugar present.		Loss.	
Before brominization.	After brominization.		
gm.	*gm.*	*gm.*	*per cent*
0.296	0.256	0.040	13.5

CONCLUSIONS.

The mixed melting point and the mutarotation undoubtedly classify the pentose in the present case of pentosuria in the *d*-xylose group. The rotation of the sugar excludes *d*-xylose; it must therefore be either *l*-lyxose or *d*-xyloketose. The ketose tests point toward the identification of this pentose as *d*-xyloketose.

Acknowledgment is due to Dr. P. A. Levene for testing the purity of the osazones obtained and for suggesting some of the chemical procedures.

BIBLIOGRAPHY.

1. Salkowski, E., *Z. physiol. Chem.*, 1899, xxvii, 507.
2. Neuberg, C., *Ber. chem. Ges.*, 1900, xxxiii, 2243.
3. Blumenthal, F., *Deutsch. Klin.*, 1903, iii, 305.
4. Rosenfeld, F., *Med. Klin.*, 1906, ii, 1041.
5. Schüler, L., *Berl. klin. Woch.*, 1910, xlvii, 1322.
6. Adler, O., and Adler, R., *Arch. ges. Physiol.*, 1905, cx, 625.
7. Blum, F., *Z. klin. Med.*, 1906, lix, 244.
8. Elliott, J. H., and Raper, H. S., *J. Biol. Chem.*, 1912, xi, 211.
9. Luzzatto, R., *Beitr. chem. Phys. u. Path.*, 1905, vi, 87.
10. Klercker, K. O., *Deutsch. Arch. klin. Med.*, 1912, cviii, 277.
11. Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1914, xviii, 319.
12. Zerner, E., and Waltuch, R., *Monatsh. Chem.*, 1913, xxxiv, 1639; 1914, xxxv, 1025.
13. Fischer, E., *Ber. chem. Ges.*, 1894, xxvii, 2486. Fischer, E., and Ruff, O., *ibid.*, 1900, xxxiii, 2142.
14. Bang, I., *Biochem. Z.*, 1915–16, lxxii, 101.
15. Van Ekenstein, W. A., and Blanksma, J. J., *Chem. Weekbl.*, 1914, xi, 189; abstracted in *Chem. Zentr.*, 1914, lxxxv, 965.
16. Neuberg, C., *Ber. chem. Ges.*, 1902, xxxv, 2628.
17. Pinoff, E., and Gude, K., *Chem. Zeit.*, 1914, xxxviii, 625.
18. Hiller, A., *J. Biol. Chem.*, 1917, xxx, 125.

THE DECOMPOSITION OF PROTEIN SUBSTANCES THROUGH THE ACTION OF BACTERIA.

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(Received for publication, February 20, 1917.)

INTRODUCTION.

The function of bacteria in the production of ammonia from the protein substances in soil is well known. How these proteins are acted upon, whether it is a hydrolytic process brought about by enzymatic action, whether an oxidative one, or whether a more complex chemical reaction, is yet an unsettled question. In order to ascertain, if possible, by controlling influencing factors as much as conditions would permit, the chemical changes that proteins undergo when acted upon by bacteria, the cause of partial ammonification of protein substances, and the nature of the reactions in general, the following series of experiments was undertaken.

EXPERIMENTAL.

Series I.

In order to follow out a system by which absolute control of influencing factors might be governed, protein substances as pure as possible were selected for the source of nitrogen and mixed with acid-treated and washed Ottawa sand instead of soil. The following proteins were used: blood fibrin, egg albumin, and peptone. The blood fibrin was the pure substance secured from Eimer and Amend; the peptone was the imported Witte-Rostock brand; the albumin was prepared in this laboratory from the whites of eggs. Instead of using a soil infusion which might contain many varieties of bacteria and protozoa, pure organisms were prepared by the Department of Bacteriology of this Experiment Station

and the sand cultures were inoculated with the same. The following organisms, which are generally recognized as good ammonifiers, were used: *Bacillus subtilis*, *Bacillus mycoides*, and *Bacillus vulgaris*. The optimum moisture content of the Ottawa sand for these bacteria was ascertained so that the greatest possible activity of the bacteria might be obtained.

It has been shown by Bainbridge[1] and corroborated by the recent work of Sperry and Rettger[2] that pure native proteins are not acted upon by bacteria, and the presence of a synthetic solution containing the necessary food elements is necessary. Although all the proteins used in this work are not considered native proteins, in order to control experiments alike, 10 cc. of the following synthetic solution were added to each culture.

	gm.
Mannite	20.0
K_2HPO_4	0.5
$CaCl_2$	0.2
$MgSO_4$	0.2
$FeCl_3$ (10 per cent solution)	0.2 cc.
H_2O to make	1,000.0 "

Each of the experiments was carried out as follows: 50 gm. of the Ottawa sand were introduced into each of several 250 cc. Erlenmeyer flasks and 1 gm. of the blood fibrin, albumin, or peptone was added and thoroughly mixed with the sand. 10 cc. of the synthetic solution were next added, together with enough distilled water to obtain optimum moisture content. The flasks were then sterilized and each was inoculated with one type of the pure cultures, *Bacillus subtilis*, *Bacillus mycoides*, or *Bacillus vulgaris*, placed in the incubator, and allowed to ammonify for 2 weeks at 28–30°C.

During the incubation period some ammonia is volatilized and will be lost unless proper precautions for its retention are provided. In these experiments the collecting of the ammonia volatilized was accomplished as follows: Each of the culture flasks was connected with a flask containing 0.1 N sulfuric acid and to a second flask holding a dilute acid (H_2SO_4) solution. Throughout the incubation period air was slowly drawn, first through the dilute

[1] Bainbridge, F. A., *J. Hyg.*, 1911, xi, 341.

[2] Sperry, J. A., and Rettger, L. F., *J. Biol. Chem.*, 1915, xx, 445.

acid solution, which served to remove the ammonia from the atmosphere and to furnish sufficient moisture to prevent excessive evaporation from the cultures, then through the culture flasks, and finally into the flasks containing the standard acid.

Ammonification was allowed to continue for 2 weeks when incubation was checked and a quantitative determination made of the various forms of nitrogen obtained from the acid hydrolysis of the protein. The determinations made consisted of total nitrogen, amide nitrogen, humin nitrogen, diamino-acid nitrogen, monoamino acid nitrogen, and ammonia. The ammonia that was collected in the standard acid during incubation was determined by titrating the excess acid. That which remained in the culture was determined as follows: To the mixture of sand and protein was added sufficient ammonia-free water to permit the protein to become suspended while the heavier sand settled to the bottom of the flask. The water containing suspended protein could then be decanted off. This operation was repeated until most of the protein was removed from the sand. The small amount which remained was obtained by adding a 15 per cent solution of hydrochloric acid and heating upon the steam bath until protein was sufficiently hydrolyzed and could be washed out of the sand. The ammonia contained in the aqueous solution was then determined by making slightly alkaline with lime water and collecting the ammonia in standard sulfuric acid by means of aeration. After the ammonia had been removed, concentrated hydrochloric acid was added to the water solution until a specific gravity of 1.15 was obtained, and the protein was hydrolyzed by heating under a reflux condenser for 72 hours.

The greater part of the acid was removed from the solution resulting from the hydrolysis by evaporating at boiling temperature to small volume. The solution which remained was transferred to a volumetric flask of 250 cc. capacity, made up to volume with water, and aliquot portions were taken for the determination of the various forms of nitrogen.

The Van Slyke method[3] for the determination of the forms of nitrogen in hydrolyzed protein was used in this work. A few slight modifications were made to adapt the method to the work in hand.

[3] Van Slyke, D. D., *J. Biol. Chem.*, 1911–12, x, 15.

To secure proper data for comparison, the different forms of nitrogen in each of the original proteins were ascertained by taking 1 gm. samples, hydrolyzing, and making the same determinations.

The results of this series of experiments are given in Tables I, II, and III. To afford means of comparison the amounts of the

TABLE I.

Results Obtained with Blood Fibrin.

Organism.	Total N.	Ammonia N.	Amide N.	Humin N.	Diamino-acid N.	Mono-amino-acid N.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Blank	146.9		17.5	4.55	33.6	95.9
Bacillus mycoides	103.6	72.7	17.5	6.65	22.8	58.8
" *subtilis*	92.8	63.3	21.7	9.30	15.0	46.2
" *vulgaris*	149.1	10.8	19.7	11.20	31.8	86.6
Decomposed.						
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Blank	—		—	—	—	—
Bacillus mycoides	29.6		—	31.3+*	32.2	38.7
" *subtilis*	36.8		24.0+	102.1+	55.4	51.9
" *vulgaris*	—		12.6+	143.3+	5.4	9.5

* + indicates an increase in that form of nitrogen.

TABLE II.

Results Obtained with Egg Albumin.

Organism.	Total N.	Ammonia N.	Amide N.	Humin N.	Diamino-acid N	Mono-amino-acid N.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Blank	141.4	—	15.4	3.8	25.9	98.0
Bacillus mycoides	88.9	55.3	15.5	9.4	23.4	46.2
" *subtilis*	90.3	57.5	18.8	3.7	12.9	56.0
" *vulgaris*	123.1	13.3	12.1	3.5	23.3	84.0
Decomposed.						
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Blank	—		—	—	—	—
Bacillus mycoides	37.1		0.6+	143.5+	9.3	57.8
" *subtilis*	36.1		22.0+	5.1	50.0	43.0
" *vulgaris*	12.9		21.4	10.2	10.0	14.3

TABLE III.

Results Obtained with Peptone.

Organism.	Total N.	Ammonia N.	Amide N.	Humin N.	Diamino-acid N.	Mono-amino-acid N.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Blank	153.7	—	15.6	4.4	36.1	98.0
Bacillus mycoides	89.6	78.3	23.2	6.6	22.4	38.0
" *subtilis*	71.4	77.7	28.0	2.6	11.3	30.1
" *vulgaris*	130.6	20.6	19.6	4.3	31.1	75.6

Decomposed.

	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Blank	—		—	—	—	—
Bacillus mycoides	41.8		48.7+	48.9	38.0	61.3
" *subtilis*	46.4		79.5	40.9	68.7	69.3
" *vulgaris*	15.1		25.6+	2.3	13.8	22.9

different forms of nitrogen are given, both in terms of mg. present and percentages of the different forms of nitrogen decomposed.

Inspection of the tables shows clearly that there is a marked difference in the action of each organism upon the proteins used. Furthermore, none of the organisms tried acts to a greater extent upon all the proteins than the others; on the contrary, an organism which ammonifies one protein rapidly may ammonify another protein comparatively slowly. In general, the results indicate that the rapidity and extent to which a protein is ammonified in a certain length of time depend upon the organism used as the acting agent. Thus *Bacillus subtilis* ammonifies the nitrogen of blood fibrin 36.8 per cent and *Bacillus mycoides* 29.6 per cent, while *Bacillus vulgaris* shows but little ammonification during the time allowed for incubation. On the other hand, albumin is ammonified more by *Bacillus mycoides* than by either *Bacillus subtilis* or *vulgaris*. In this protein, however, it is to be noted that *Bacillus vulgaris* ammonified it to the extent of 12.9 per cent. Again, in the case of peptone, *Bacillus subtilis* is the most rapid ammonifying organism, changing 46.4 per cent of its nitrogen into ammonia, *Bacillus mycoides* being second with 41.8 per cent, and *Bacillus vulgaris* least with 15.1 per cent.

The monoamino-acid nitrogen and diamino-acid nitrogen are the forms which appear to be most readily changed. The extent

of transformation varies in the different proteins, depending upon the bacteria used, being greater in the monoamino-acid nitrogen in one case, while in another protein the diamino-acid nitrogen will show a higher percentage.

The humin nitrogen and amide nitrogen constitute only a small percentage of the total nitrogen. Nevertheless, changes worthy of consideration are shown in these fractions. *Bacillus mycoides* causes a consistent increase in the humin nitrogen in all the proteins used, and in the case of blood fibrin all the organisms effected an increase of this form. Work by several investigators indicates that this increase may be due to the presence of a sugar since this substance was employed in the preparation of the synthetic solution. Amide nitrogen also showed an increase in nearly all cases which can be explained only on the basis that there was an actual synthesis of this form.

Series II.

In order to gain a deeper insight relative to the changes that occur during the process of ammonification, a separation of the water-soluble and insoluble portions of the ammonified protein was attempted. This was accomplished by centrifuging the water solution of protein substance after separating from the sand as described in the preceding series. The solid or insoluble material thus obtained was hydrolyzed by boiling with strong hydrochloric acid and one-half the soluble portion was treated in like manner. Determinations of the various forms of nitrogen were made in these hydrolyzed samples, together with the other portion left unhydrolyzed.

The details of the preparation of the cultures were similar to the preceding series. Casein and gliadin were the proteins employed. *Bacillus subtilis* was the only type of bacteria used and a double number of cultures were prepared in order that the period of incubation might be extended over two different lengths of time. Thus, for casein, one culture was allowed to ammonify for 6 days and the other for 8 days, while gliadin, which ammonifies very slowly, was allowed to proceed for 15 and 30 days. The casein and gliadin used were prepared in this laboratory, the former from cow's milk and the latter from the gluten of wheat flour.

TABLE IV.

Results Showing Changes in the Different Forms of Nitrogen during Ammonification of Gliadin by the Organism Bacillus subtilis.

Experiment.		Total N.	Amide N.	Humin N.	Diamino-acid N.	Monoamino-acid N.	Ammonia N.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Blank		100.0	25.92	1.84	4.0	68.6	
After 15 days.	Insoluble matter.	22.24	4.64	1.04	2.40	14.08	51.60
	Soluble "	26.32	9.84	1.65	2.24	13.12	
After 30 days.	Insoluble matter.	11.04	1.84	1.04	0.88	8.88	63.84
	Soluble "	24.24	6.96	4.40	3.38	11.12	
Decomposed.							
15 days		51.44	44.13	47.82+	16.0+	60.37	
30 "		64.72	66.05	195.65+	6.0+	70.86	

TABLE V.

Results Showing Changes in the Different Forms of Nitrogen during Ammonification of Casein by the Organism Bacillus subtilis.

Experiment.		Total N.	Amide N.	Humin N.	Diamino-acid N.	Monoamino-acid N.	Ammonia N.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Blank		100.0	12.71	2.29	17.75	68.60	
After 6 days.	Insoluble matter.	16.13	2.61	2.38	2.53	9.68	45.02
	Soluble "	38.71	15.86	1.69	4.84	17.67	
After 8 days.	Insoluble matter.	16.08	2.68	0.62	3.05	10.18	45.02
	Soluble "	38.05	18.75	1.45	4.72	13.93	
Decomposed.							
6 days		44.56	45.18+	76.66+	58.62	60.26	
8 "		45.86	68.67+	10.00	56.03	64.84	

The results obtained above substantiate, in general, those of the preceding series. All the forms of the nitrogen in the protein used are changed or acted upon by *Bacillus subtilis*. Since there is but a small amount of diamino-acid nitrogen in casein and gliadin, the monoamino-acid nitrogen was evidently the source of most of the nitrogen for the ammonia formed. A comparison of

the rate of ammonification of different proteins shows that they may vary widely, casein and gliadin being extreme examples. Casein attained maximum ammonification in 6 days, and probably before that time, as has been shown by Kelley,[4] while gliadin had not reached the maximum point after 30 days.

It is noted that a greater percentage of all the different forms of nitrogen is contained in the soluble portions, although before bacterial action very little of either casein or gliadin is soluble. The changes in the different forms of nitrogen, soluble and insoluble, are evident from the tables.

The half of the soluble portion that was not hydrolyzed was used to make determinations of the soluble amino nitrogen. This was accomplished by taking an aliquot part and analyzing by the Van Slyke method, using the small Van Slyke apparatus. Similar determinations were made upon acid-hydrolyzed samples and water solutions of the untreated protein. The water solution was prepared by taking 1 gm. of the protein substance, adding 20 cc. of distilled water, and allowing it to stand 5 hours, shaking at frequent intervals. After allowing to settle for a few minutes, the supernatant liquid was decanted through filter paper.

Table VI shows a comparison of the amount of free amino-acid nitrogen contained in gliadin and casein before and after hydrolysis and after ammonification by the organism *Bacillus subtilis*.

TABLE VI.

	Before hydrolysis.	After hydrolysis.	After ammonification.
	mg.	*mg.*	*mg.*
Gliadin	0.57	42.56	17.03
Casein	0.68	99.31	46.00

We note from these results that there was a very small amount of free amino nitrogen in the water solution of the untreated protein, while in the acid-hydrolyzed and ammonified sample a large amount was obtained. This indicates a similarity of chemical change between acid hydrolysis and bacterial action, and we would therefore infer that in ammonification the protein is hydrolyzed into amino-acids by the action of bacteria.

[4] Kelley, W. P., *Hawaii Agric. Exp. Station Bull. 37*, 1915.

The results obtained thus far do not lead to any definite conclusions relative to the cause of incomplete ammonification of protein substances. All the various forms of nitrogen are present after bacterial action, although necessarily in different proportions. The question naturally arises: Can it be the presence of a toxic substance resulting from bacterial decomposition that tends to inhibit ammonification beyond a certain point? For the purpose of getting light upon this question, sand cultures of casein were prepared as in the previous experiment, sterilized, inoculated with *Bacillus subtilis*, and allowed to incubate until no further ammonification was noticed. The unammonified protein substance was then separated from the sand and a further separation of the insoluble and soluble material, as described above, was effected. These separate parts were then dried on the steam bath, ground to a fine powder in a mortar, introduced into individual flasks containing fresh sand, sterilized, and again inoculated with a fresh culture of *Bacillus subtilis*. Ammonification was allowed to continue first for 2 weeks, after which time a very small amount of ammonia was formed. The flasks were again placed in the incubator and allowed to ammonify for 3 weeks longer when analysis showed that the ammonia continued to be formed only in negligible amounts. During this time, the organism, *Bacillus subtilis*, was the only organism present; and in order to ascertain whether or not other bacteria, as are present in an ordinary soil infusion, would show a greater decomposition of the remaining protein substance, with the ultimate formation of ammonia, the flasks were now inoculated with a soil infusion and allowed to ammonify for 2 weeks. The results indicated a slight ammonification, yielding 2.4 mg. of ammonia for the flask containing the solid material and 2.1 mg. for the other containing the solution. The amount of ammonia formed in 2 weeks is very small, and after 5 weeks' further incubation it increased slightly.

The results indicate that the degree of ammonification is not influenced by the presence or absence of any particular form of nitrogen contained in the protein molecule, nor by any decomposition product which could be destroyed by sterilization, nor by the presence of a toxic substance. If these inferences are true, it is probable that ammonification can proceed only to a point where there occurs a residual portion of the original molecule

which is sufficiently stable to withstand the action of bacteria. Any action that would be sufficiently strong to tear down this residue would in all probability permit further ammonification. Whether this further cleavage of the protein molecule occurs under soil conditions is a matter which can only be determined by further experiment.

SUMMARY.

A limited study has been made on the chemical changes that occur when a protein substance is acted upon by certain organisms.

The results indicate that:

1. All the nitrogen forms are changed more or less by the action of the bacteria, and the end-product ammonia is formed. In no case was one form of nitrogen completely destroyed.

2. The rapidity of action varies greatly with different protein substances, casein showing no further change after a few days, while gliadin continued to ammonify after 30 days.

3. One organism does not act upon different proteins alike. The relative proteolytic activities of the organisms used depend on the proteins acted upon.

4. The monoamino-acid nitrogen and diamino-acid nitrogen of the protein are the chief sources of the ammonia formed by bacterial action.

5. The similarity of chemical change between the action of acid hydrolysis and the action of bacteria indicates that the latter is largely hydrolysis to the point of formation of various amino-acids.

6. There is no toxic substance formed that would inhibit complete ammonification of a protein.

THE EFFECT OF THE INGESTION OF DESICCATED PLACENTA ON THE VARIATIONS IN THE COMPOSITION OF HUMAN MILK DURING THE FIRST ELEVEN DAYS AFTER PARTURITION.

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INTRODUCTION.

In order to arrive at any conception of the factors underlying mammary secretion it is necessary to differentiate between the period of development of the mammæ, the period of their hypertrophy during pregnancy, and the period of secretory activity after parturition. From the standpoint of biochemistry the third period is one of special importance.

The influence of the placenta on the secretion of milk has been the object of attention of several investigators. Fellner (1) and Biedl and Königstein (2) seem to think that repeated injections of placental extract tend to cause hypertrophy of the gland, yet neither Kreidl and Mandl (3) nor Frank and Unger (4) could observe this effect. From the researches of O'Donoghue (5, 6, 7) and of Ancel and Bouin (8) it is evident that the internal secretion of the ovaries is the most important factor in the preparation of the mammary gland for the assumption of its function.

Niklas (9) gives a comprehensive bibliography of the observations on the effect of placenta on milk secretion from the quantitative standpoint. Basch (10), Lederer and Pribram (11), Aschner and Grigoriu (12), and Niklas (9) claim that an increased secretion of milk usually occurred as the result of the injections of placental extract. Fieux (13), Lane-Claypon and Starling (14), Biedl and Königstein (2), Mackenzie (15), and Gaines (16) fail to find such an increase. In fact both Mackenzie and Gaines report an inhibition. Feeding experiments have given similar inconstant results. On women the increase was usually of but slight degree only.

Letulle and Larrier (17) consider that the active substance came from the syncytium, while Ercolani (18), Creighton (19), and Pinoy (20) claim that it is the result of the degenerative changes in the placenta.

As the natural result of these observations there are two sets of ideas regarding the part played by the placenta in milk secretion. The one initiated by Hildebrandt (21) and supported by Halban (22) rests upon the negative results, and considers that during pregnancy the placenta gives off a substance inhibitory to the secretory activity of the mammary gland, but excitatory as regards its hypertrophy, with the complementary idea that the removal of this inhibitory stimulus results in secretory activity. A chalone action is thus attributed to the placental secretion. The other, probably best expressed by Niklas, considers that at delivery there is an overflowing of hormones into the maternal blood, which after a certain period of incubation bring about the initiation of the activity of the milk-producing cells, and which in all probability come from the placenta.

It would appear from the foregoing that a chemical study of the composition of human milk when produced under the influence of placental ingestion would be of some assistance in determining whether or not the activity partakes of the type of a chalone or hormone. It would likewise show in some degree the influence of the placenta upon the maternal metabolic changes preparatory to the taking on of a food-producing function.

In a previous paper (23) a somewhat detailed report was given of the variations in the chemical composition of human milk during the first 11 days after parturition. The present paper is a report of the changes induced in these variations by the oral administration of desiccated placenta.

The subjects of this experiment were healthy patients in the Los Angeles County Hospital. Throughout the period of study the diet was the same for all, and was the same as for those previously reported, with the exception that 0.6 gm. of desiccated placenta was ingested three times a day, commencing with the day of delivery. The milk production of eight women was studied.

Case.	Date.	Case.	Date.
	1916		*1917*
1	Aug. 28–Sept. 5	5	Feb. 10–18
2	" 29– " 6	6	" 13–21
3	" 29– " 6	7	" 13–21
4	" 30– " 7	8	" 25–Mar. 5

The milk was collected by means of the breast pump at the same hour on the 3rd, 5th, 7th, 9th, and 11th, days after parturition.

Methods.

Preparation of the Placenta.—The fresh placenta was ground to a pulp in a meat grinder, mixed with 5 to 10 cc. of toluene, and spread in a thin coat over a large glass plate. It was then dried, without decomposition, in about 48 hours, to a leathery, semi-brittle mass, using the air current from an electric fan. This material was then ground as fine as possible and dried for 24 hours over sulfuric acid in a partial vacuum at room temperature. Regrinding resulted in a fine dry powder of uniform consistency and a not unpleasant odor. This powder was administered in capsules.

Analyses.—The analytical processes used were the same as those given in the report on normal milk secretion (23).

Total Nitrogen and Protein.

Total nitrogen only was determined and the protein calculated from these figures by multiplying by the factor 6.37. With the exception of the 3rd day the ingestion of placenta has the effect of increasing the per cent of protein in the milk about 12 per cent. It apparently causes a lowering of the initial protein per cent in colostrum. The results are recorded in Table I.

TABLE I.

The Percentage of Protein in Human Milk on the 3rd, 5th, 7th, 9th, and 11th Days after Parturition, when Desiccated Placenta Is Fed.

Subject.	1.	2.	3.	4	5.	6.	7.	8.	Average.	High.	Low.
Day.	Protein.										
3rd	2.11	6.08	3.44	1.90	2.22	2.24	2.32	2.68	2.87	6.08	1.90
5th	1.64	1.85	2.52	1.56	1.82	1.79	2.22	2.09	1.94	2.52	1.56
7th	1.55	1.83	2.87	1.86	1.72	1.60	2.06	1.77	1.91	2.87	1.55
9th	1.41	1.86	2.95	1.90	1.52	1.93	2.12	1.70	1.92	2.95	1.41
11th	1.20	1.82		1.42	1.61	1.38	2.02	1.33	1.54	2.02	1.20
Average.	1.58	2.69	2.95	1.73	1.78	1.79	2.15	1.91	2.04	3.29	1.52

Group Variations for Each Day.—In 88 per cent of the cases on the 3rd day the extreme limit of variation from the mean is ± 29 per cent, which is 40 per cent less variation than that found when no placenta is fed. This limit undergoes a slight narrowing on the 5th day, but from then on expands, so that by the 11th day of lactation the variation from the mean is ±25 per cent, almost equal to the initial limit and nearly 40 per cent greater than normal.

Group Variations from Day to Day.—From the 3rd to the 11th day of lactation there is a general fall in protein production, the most marked decline taking place between the 3rd and 5th days. It should be noted that, as with normal protein production, there is also here an evident tendency towards an increase in protein per cent between the 7th and 9th days. This increase is of sufficient frequency to denote the presence of some important change taking place in the protein production mechanism at this time. Qualitatively, therefore, the ingestion of placenta fails to alter the usual course of production direction. Quantitatively the decrease in production is of a lesser degree than normal, resulting in a uniformly higher protein per cent in the milk of women ingesting the desiccated placenta.

The Effects of the Ingestion of Desiccated Placenta on the Variations in the Protein Percentage of Human Milk.—The feeding of desiccated placenta to women during the first 11 days of lactation decreases the per cent of protein in colostrum; it increases the production from then on about 12 per cent above the normal; it causes a narrowing of the limits within which this production may be supposed to occur in colostrum, with an evident later expansion of these limits above those normally found; it does not alter the normal changes in production direction at any time; it has practically no effect on the individual variability from day to day; and the plane of protein production remains nearly as uniformly above or below the group average for the single days as when no placenta is ingested.

Fat.

In Table II will be found the per cent of fat in the milk of eight women on the 3rd, 5th, 7th, 9th, and 11th days after parturition, when desiccated placenta is fed.

As a whole, the effect of the ingestion of desiccated placenta on the fat per cent in human milk is the opposite of that produced on the protein. It causes a tendency towards an increase in the per cent of fat in colostrum, with a decrease in the succeeding days, when compared with the milk produced by individuals not subjected to its influence.

The Effects of the Ingestion of Desiccated Placenta on the Variations in the Fat Percentage of Human Milk.—The feeding of desiccated placenta to women during the first 11 days of lactation tends to decrease the per cent of fat in milk; it causes an expansion of

TABLE II.

The Percentage of Fat in Human Milk on the 3rd, 5th, 7th, 9th, and 11th Days after Parturition, when Desiccated Placenta Is Fed.

Subject.....	1.	2.	3.	4.	5.	6.	7.	8.	Average.	High.	Low.
Day.	Fat.										
3rd	2.26	7.06	3.32	4.14	3.72	2.88	4.88	6.53	4.35	7.06	2.26
5th	1.44	2.94	1.89	2.57	3.89	3.08	4.80	2.30	2.58	4.80	1.44
7th	2.10	3.30	4.41	2.59	2.14	3.69	8.88	2.67	3.72	8.88	2.10
9th	4.09	2.36	3.10	1.74	1.93	2.46	4.84	3.52	3.00	4.84	1.74
11th	2.72	3.98		2.71	2.63	3.62	6.30	4.17	3.73	6.30	2.63
Average.	2.52	3.93	3.18	2.75	2.86	3.15	5.94	3.84	3.48	6.38	2.03

the limits within which the production may be supposed to occur in colostrum; it causes a later narrowing of these limits below those normally found; it tends to regulate the variation in production direction; it increases the individual variability to a marked extent; and it causes the plane of fat production to tend towards uniformity to a greater degree than is found in the milk of individuals not subjected to its influence.

Lactose.

In Table III will be found the per cent of lactose in the milk of eight women on the 3rd, 5th, 7th, 9th, and 11th days after parturition, when desiccated placenta is fed.

TABLE III.

The Percentage of Lactose in Human Milk on the 3rd, 5th, 7th, 9th, and 11th Days after Parturition, when Desiccated Placenta Is Fed.

Subject.....	1.	2.	3.	4.	5.	6.	7.	8.	Average.	High.	Low.
Day.	Lactose.										
3rd	6.49	6.37	5.74	5.16	4.47	5.73	5.88	5.83	5.71	6.49	4.47
5th	7.00	6.20	6.49	6.81	5.47	6.12	6.26	6.63	6.37	7.00	5.47
7th	7.19	7.00	4.05	7.03	6.56	6.44	5.98	6.56	6.35	7.19	4.05
9th	7.53	7.14	5.58	7.20	6.66	6.69	5.75	5.98	6.57	7.53	5.58
11th	7.37	7.03		7.24	6.86	6.86	6.01	5.85	6.75	7.37	5.85
Average.	7.12	6.75	5.47	6.69	6.00	6.37	5.98	6.17	6.35	7.12	5.08

A comparison of the per cent of lactose found in the milk of women during the first 11 days of lactation, with and without the ingestion of placenta, will demonstrate that in the former there has been a consistent increase in sugar production.

The Effects of the Ingestion of Desiccated Placenta on the Variations in the Lactose Percentage of Human Milk.—The feeding of desiccated placenta to women during the first 11 days of lactation causes an increased activity of the lactose-producing mechanism with the consequent generally higher sugar per cent in the milk; it does not alter to an appreciable extent the variability of the limits within which this production may be supposed to occur; although decreasing the individual variability, it seems to cause a slight instability of the usual normal uniformity of change in production direction, an effect opposite to that observed on fat; it causes a diminution of the normal tendency towards a uniformity of plane of production, again different from what occurs with fat.

Variations in the Constituents.

With the decrease in the plane of production for fat there is an increase in the plane of production for both protein and lactose when these results are compared with those obtained during normal milk secretion. The reciprocal relationship existing between fat and lactose production in normal milk when calculated from the individual per cent variation from day to day and the per cent change in production direction is not as well marked under the influence of ingested desiccated placenta. This is due in part to the decrease of the uniformity of change in production direction, and in part to the decrease in per cent variability from day to day, of the lactose production. The alteration in the plane of production of these two constituents seems to have a tendency towards compensating their individual reciprocal variability, which, however, does not in the least signify that they are not still mutually interdependent.

SUMMARY.

In comparing the variations in the fat, protein, and lactose per cent of milk produced during the first 11 days after parturition, when desiccated placenta is fed, with the variations observed

in these constituents for a similar period in normal milk, it is seen that there are some significant differences. Quantitatively the plane of production for lactose and protein has been raised, while that for fat has been slightly decreased. The general change in production direction remains the same.

When we consider as an index of variability the individual variation per cent from the group average for the single days and the individual variation per cent from day to day, the constituents fall into the same order of decreasing variability as in normal milk production; *fat, protein, lactose.* But when the group changes in variation limits and the group uniformity of change in production direction are considered, the order changes from *fat, lactose, protein,* as found in normal milk, to *lactose, fat, protein,* with but slight differences between them.

The tendency for the production plane to be fixed for the individual and independent of the plane of nutrition is here maintained, though to a slightly less extent.

CONCLUSIONS.

The results recorded in this paper demonstrate that the ingestion of desiccated placenta has an effect upon the factors concerned in the regulation of the chemical composition of milk. There is a stimulation of the sugar- and protein-producing mechanism with an apparent depression of the function of the fat-secreting apparatus.

From the peculiar characteristics of milk protein and carbohydrate it is presumable that these constituents are largely elaborated by the gland itself.

There is certain evidence pointing to the idea that the milk fat is not entirely elaborated in the mammary gland. In working with goats, Winternitz (24, 25) and Bowes (26) have shown that probably a part of the ingested fat is carried as such to the mammary gland and then by excretion becomes a milk constituent. Eckles and Palmer (27, 28) clearly show that for the cow there is a variation in the nature and composition of milk fat dependent in some degree upon the diet. The probability of there being present in the gland a specific fat production mechanism for the elaboration of the fats peculiar to milk cannot be denied even though Bradley (29) failed to detect the presence of a lipase in

this tissue. It would therefore appear that milk fat is the sum total of the secretory and excretory activities of the mammary gland, the former being concerned with the elaboration of the fat peculiar to milk, the latter concerned in the inclusion in the milk of a part of the ingested fat as such. The evidence for this is admittedly incomplete.

From the fact that the ingestion of desiccated placenta tends to produce a milk of greater uniformity in change of production direction of fat, it does not seem improbable that its action may well be stimulative to the secretory activity of the gland in this respect also. The cause of the lower per cent of fat in the milk may indeed be founded either in a decreased absorption of ingested fat or in a decreased fat excretory activity of the gland.

These findings seem to lend support to the hypothesis of Niklas (9).

No chalone activity is evident.

BIBLIOGRAPHY.

1. Fellner, O. O., *Arch. Gynaek.*, 1913, c, 641.
2. Biedl, A., and Königstein, R., *Z. exp. Path. u. Ther.*, 1910–11, viii, 358, cited in Fellner (1) and Niklas (9).
3. Kreidl and Mandl, *Wien. klin. Woch.*, 1905, xviii, 73.
4. Frank, R. T., and Unger, A., *Arch. Int. Med.*, 1911, vii, 812.
5. O'Donoghue, C. H., *J. Physiol.*, 1911, xliii, p. xvi.
6. O'Donoghue, *Quart. J. Micr. Sc.*, 1911, n.s. lvii, pt. 2, 187.
7. O'Donoghue, *J. Physiol.*, 1913, xlvi, p. vi.
8. Bouin, P., and Ancel, P., *J. physiol. et path. gén.*, 1910, xii, 1.
9. Niklas, F., *Monatschr. Geburtsh. u. Gynaek.*, 1913, xxxviii, 60.
10. Basch, K., *Deutsch. med. Woch.*, 1910, xxxvi, 987.
11. Lederer, R., and Pribram, E., *Arch. ges. Physiol.*, 1910, cxxxiv, 531.
12. Aschner and Grigoriu, *Arch. Gynaek.*, xciv, pt. 3, cited in Fellner and Niklas.
13. Fieux, G., *Bull. méd.*, 1903, xvii, 725, cited in Fellner and Niklas.
14. Lane-Claypon, J. E., and Starling, E. H., *Proc. Roy. Soc., Series B*, 1905–06, lxxvii, 505.
15. Mackenzie, K., *Quart. J. Exp. Physiol.*, 1911, iv, 305.
16. Gaines, W. L., *Am. J. Physiol.*, 1915, xxxviii, 285.
17. Letulle, M., and Larrier, L., *Rev. de gynéc.*, 1901, cited in Fellner.
18. Ercolani, cited in Fellner, p. 670.
19. Creighton, cited in Fellner, p. 670.
20. Pinoy, *Bull. Soc. biol.*, 1900, cited in Fellner.
21. Hildebrandt, P., *Beitr. chem. Phys. u. Path.*, 1904, v, 463.

22. Halban, J., *Arch. Gynaek.*, 1905, lxxv, 353, cited in Fellner and Niklas.
23. Hammett, F. S., *J. Biol. Chem.*, 1917, xxix, 381.
24. Winternitz, H., *Deutsch. med. Woch.*, 1897, xxiii, 477.
25. Winternitz, *Z. physiol. Chem.*, 1898, xxiv, 425.
26. Bowes, O. C., *J. Biol. Chem.*, 1915, xxii, 11.
27. Eckles, C. H., and Palmer, L. S., *Missouri Agric. Exp. Station Research Bull. 24*, 1916.
28. Eckles and Palmer, *Missouri Agric. Exp. Station Research Bull. 25*, 1916.
29. Bradley, cited in Mathews, A. P., Physiological Chemistry, New York, 2nd edition, 1916, 313.

STUDIES ON THE THEORY OF DIABETES.

VIII. TIMED INTRAVENOUS INJECTIONS OF GLUCOSE AT LOWER RATES.*

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INTRODUCTION.

Principles underlying the method of timed intravenous injections at known rates, and an improved pump designed for the purpose were recently described.[1] Their application in a clinical study of glucose tolerance has been reported elsewhere by Wilder and Sansum.[2] This paper deals generally with the technique of the experiments carried out on rabbits and dogs, and, in particular, with observations concerning the effects of sustained intravenous injections of glucose at rates up to 2 gm. per kg. per hour. The experiments with higher rates of injection will be made the subject of another paper. Apart from recording certain data concerning the behavior of glucose and water, the purpose of this paper is further to discuss the relative importance of rate and concentration measurements in biological work generally.

Methods.

Material.—The pure glucose used in the majority of experiments was prepared in the laboratory as described below from a completely hydrolyzed granular commercial glucose from cornstarch.[3] A Kahlbaum c. p. glucose preparation was used at in-

* A third detailed report on phases of the work outlined by Woodyatt, R. T., Sansum, W. D., and Wilder, R. M., *J. Am. Med. Assn.*, 1915, lxv, 2067.

[1] Woodyatt, *J. Biol. Chem.*, 1917, xxix, 355.

[2] Wilder and Sansum, *Arch. Int. Med.*, 1917, xix, 311.

[3] Made at the Argo plant of The Corn Products Refining Company.

tervals for control purposes. Some lots of the product made in the laboratory caused melituria when injected at rates below that found necessary with the Kahlbaum preparation.

1,000 gm. of the crude glucose are dissolved in 400 cc. of boiling water digested 1 to 2 hours (not longer) with animal charcoal and the solution filtered hot by suction. Add 800 cc. of 95 per cent alcohol, cool, and inoculate with glucose crystals. The first crystals (5 to 10 gm.) carry down suspended carbon and are filtered off. As further crystallization proceeds, filter off successive fractions to avoid caking. Wash the crystals with cold alcohol on a suction funnel. (Save the alcoholic washings to dilute the next concentrated crude glucose filtrate.) Yield = 50 to 60 per cent. Repeat the process if necessary. If the crude glucose employed contains much tarry matter, it may be necessary to use more than 400 cc. of water per 1,000 gm. at the outset to obtain a filterable solution. The final pure white product was made up into 1, 2, 3, and 4 molecular (18, 36, 54, or 72 per cent) solutions with water freshly distilled as for a salvarsan injection and placed in 2 to 3 liter flasks in which they showed only a straw or light yellow color. This color may be wholly removed by a final clearing with a little bone-black. After sterilization and before use, the exact strength of each solution was determined by polariscope checked at times by a reduction method. Solutions of 1 and 2 molecular strength proved most generally useful.

It may be noted that a 1 gm. molecular or 18 per cent aqueous glucose solution contains, at 17.5°C. per 100 cc. volume, 89.44 gm. of water (Baling) and 18 gm. of glucose, or 1 molecule of glucose to 50 molecules of water, whereas a 2 gm. molecular (36 per cent) aqueous glucose solution contains, per 100 cc., 79.90 gm. of water and 36 gm. of glucose, or 1 molecule of glucose to about 22 molecules of water.

The subjects were dogs, rabbits, and human beings. The bulk of the work was with dogs weighing from 9 to 19 kg. Care was taken to select vigorous animals of gentle and phlegmatic dispositions received from active outdoor life. Bull terriers or mongrels with a strong admixture of bulldog blood were used in most of the satisfactory experiments, whereas most of the failures occurred with unhealthy, housed, ill fed, or nervous animals.

Technique.—The technique of the experiments as applied to human subjects was the same as that described and illustrated by Wilder and Sansum[2] in their report on clinical glucose tolerance determinations by the intravenous method. The routine of the dog experiments requires further description.

The solution to be injected is poured into a previously sterilized burette of about 500 cc. capacity. The burette has a glass cock and the tip connects by rubber tubing with one arm of a glass Y-tube from the stem of

which rubber tubing leads to the suction nipple of a volumetric pump. A second slender burette beside the first connects with the other arm of the Y-tube and serves for short interval readings. Half hourly or hourly rates are checked on the reservoir burette. One or two coils of the rubber tubing which carries the solution from the burettes to the pump are allowed to rest in a water bath to warm the sugar solution. The delivery tube from the pump terminates in a small cannula inserted into a vein, usually at the ankle in the case of dogs. With rabbits the injections have been through a small needle inserted into the ear vein and held by a small clamp. In many of the experiments two pumps have been employed, the second or auxiliary taking distilled water from a second pair of burettes and discharging it through a delivery tube and a needle which is thrust obliquely through the wall of the rubber delivery tube of the main pump; or, the two delivery tubes may be permanently anastomosed. Thus it is made possible *to control the concentrations of water and glucose as well as the absolute quantities in which they are injected.*

The dogs lie on their backs or partly on the side in an ordinary animal trough cushioned with blankets. There is a leather collar about the neck and a not too snug jacket or flannel bandage on the chest. Collar and jacket are secured below to the animal board allowing limited freedom of movement, care being taken not to impede respiration. The fore legs are free. The hind legs are separated to the opposite lower borders of the board and held firmly by slings but not in such a way as to cause pain or stop circulation.

A catheter is placed in the bladder and secured by a strip of adhesive tape fixed to the catheter and carried upward on to the abdomen. It was found desirable to accustom the dogs to these procedures in sham experiments on previous days before attempting long injections. Preferably, exposure of the vein has been carried out under novocaine on the day before the experiment, in which case ligatures have been laid, the wound smeared with vaseline, and the whole enclosed with a band of adhesive tape. In the experiment the cannula may be inserted without causing pain. These precautions were taken to avoid non-physiological factors and because experience proved that restlessness, discomfort, pain, or emotion could affect the results, especially the rate of water secretion. In many experiments the laden animal board has been made to rest on the platform of a scales and the scale beam so balanced that gains or losses of body weight incident to the development of positive or negative water balances could be detected and, if desired, corrected by changing the rate of the water injection by means of the auxiliary pump.

The animal experiments have all been conducted in a separate room from which noise and confusion could be excluded. In satisfactory experiments dogs have lain quietly for many hours at a time, the pumps have required little attention, and the collection of urine has proceeded automatically except for required manipulation at the end of the hourly periods.

EXPERIMENTAL.

As an aqueous solution of glucose may be regarded as representing merely so many molecules of glucose, so many of water, or as glucose and water together, each affecting and affected by the other in a way not fully understood, to form among other things a series of hydrates and their dissociation fragments in accordance with the solvate theory,[4] so intravenous injections of aqueous glucose solutions and their physiological effects have to be considered from at least these viewpoints. Although in this report attention is centered on the glucose this necessarily involves some consideration of the corresponding movements of water into and through the body.

Continued Injections of Glucose into the Systemic Blood at Rates Not Greater Than 1.0 Gm. per Kilo of Body Weight per Hour.

In all, upwards of fifty experiments have been conducted in man, dogs, and rabbits, receiving glucose by vein at the lower rates. Conditions were considered standard when the subjects were healthy, well nourished, mature individuals in a state of complete bodily rest and free of pain, discomfort, emotion, or other tangible nerve-disturbing influence, when the glucose employed reacted chemically and physiologically as pure *d*-glucose and when the solutions employed varied in strength between 16 and 40 per cent. The gm. molecular (18 per cent) solution was most commonly used. The duration of the experiments varied from ½ hour to 7 hours.

In man, injections at the rate of 0.8 gm. per kg. per hour were not observed to produce glycosuria detectable by the ordinary test solutions employed (Fehling's, Haines'), after 30 minutes to 3 hours of injection. *In dogs and rabbits* the same results were obtained. One dog received glucose at this rate for 7 hours with the same result. Injection at the rate of 0.9 gm. per kg. per hour *in man* uniformly caused glycosuria, also *in rabbits and dogs* in the great majority of cases but not in all. In four dogs it required 1.0 gm. of glucose per kg. per hour to cause glycosuria. It

[4] Cf. Jones, H. C., Absorption spectra of solutions as studied by means of the radio-micrometer, *Carnegie Institution of Washington, Publication No. 210*, 1915. A summary of earlier literatures is given at the end.

has been observed that glycosuria has failed to develop when the injection rate has been between 0.8 and 0.85 gm., and appeared when the rate has been advanced to between 0.85 and 0.90 gm. per kg. per hour.

The time elapsing between the beginning of an injection at the 0.9 gm. rate and the detection of glycosuria was always short when this rate was instituted directly after ½ hour or more of injection at the rate of 0.6 to 0.8 gm. per kg. per hour, and longer when the 0.9 gm. rate was instituted with no prior glucose administration. Owing to the time necessary for enough urine to collect in the bladder, to remove and test satisfactorily, the exact interval elapsing between the beginning of the 0.9 gm. rate and the actual secretion of saccharine urine by the kidneys cannot be stated, but it was found to be as long as 40 minutes with one dog which had received no prior administration, and as short as 6 minutes in a dog which had received glucose for ½ hour at the 0.8 gm. rate before the rate was advanced. Following preliminary injections at a subtolerant rate for ½ hour or more, an advance to the 0.9 rate has regularly caused sugar to appear in the urine within the first half hour.

Conclusions.—On the basis of all the experiments taken collectively it is reckoned that the maximum rate at which glucose can be administered into the systemic blood and still fail to produce glycosuria in normal resting human individuals is close to 0.85 gm. per kg. per hour. Perhaps in dogs and rabbits it averages a little higher than in man. (These statements agree in the main with those made in our preliminary report on the basis of experiments then completed. They take into account the later series of observations on human subjects described by Wilder and Sansum, and further dog and rabbit observations which permit the correction of slight inaccuracies in the earlier report.)

The Behavior of Water in Conjunction with Sustained Intrasystemic Glucose Injections at Subtolerant Rates.

Two things may be noted here: (1) The same results as described above have been obtained with glucose solutions varying in concentration between 16 and 40 per cent. The method em-

ployed did not permit the detection of any difference of the intravenous glucose tolerance limit as a result of variations between rather wide limits of the quantity of water which accompanied the glucose into the vein. Thus in dogs weighing 10 kg., the limit was found at 0.85 gm. per kg. per hour both with 47 cc. of 1 M (18 per cent) and with 23.5 cc. of 2 M (36 per cent) glucose solutions. Even when solutions containing 72 to 100 gm. of glucose per 100 cc. have been used, the tolerance limit has not varied by degrees which could be detected by the methods employed. The same has held for solutions as weak as 9 per cent. However, the more concentrated solutions increase the difficulties of maintaining exact injection rates when dealing with dogs and rabbits, and following an injection of 72 per cent solution in one human case, a thrombus developed in the vein so that no further use was made of such strong solutions in the clinic. Too dilute solutions, on the other hand, may introduce such factors as hemolysis and significant changes of weight. (2) During intravenous glucose injections at subtolerant rates there has been observed a strong tendency toward retention of water in the organism, which under appropriate experimental conditions may be manifested by an absolute decrease in the rate of diuresis that appears after the injection has been continued for a sufficient length of time, lasts during the remainder of the injection, and promptly gives way to a sudden increase of diuresis when the injection is stopped. The following experiment is typical.

EXPERIMENT I.

The Retardation of Diuresis during Intravenous Injection of Glucose at a Subtolerant Rate with High Diuresis after Injection.

A dog weighing 12.7 kg. received 40 cc. of water *per os* at 8 and again at 9 a.m. when it was placed on the board, catheterized, and prepared for intravenous injection. Thereafter the bladder was emptied by catheter every hour. The injection consisted of 51.5 cc. of 19.7 per cent glucose solution (containing about 45 cc. of water) per hour. Rate, 0.8 gm. glucose per kg. per hour. The table shows the amounts of urine obtained at the end of each succeeding hour, the bold-faced figures indicating hours of injection.

Hour.	Urine.	Remarks.
	cc.	
10 a.m.	40	
11 "	30	
12 "	**13**	Injection 0.8 gm. per kg.
1 p.m.	**18**	" 0.8 " " "
2 "	**7**	" 0.8 " " "
3 "	**8**	" 0.8 " " "
4 "	**9**	" 0.8 " " "
5 "	**10**	" 0.8 " " "
6 "	**12**	" 0.8 " " "
7 "	80	

Of the 80 cc. of urine passed between 6 and 7 o'clock, 58 cc. appeared in the first 25 minutes. Thereafter for 12 hours the average urinary volume was 12 cc. per hour. It may be noted that the intake of water per hour during injection was about that given in the fore-periods. There was no glycosuria.

We have failed to observe such absolute retardations of diuresis in experiments in which the quantity of water given during the preparatory periods or during the glucose injection periods has been too great. In such cases, however, there has never been noted any increase of diuresis attributable to the glucose itself; *i.e.*, the injection of glucose solutions at subtolerant rates has not been observed to elevate the diuresis more than the injection of equivalent volumes of water in the form of 0.6 to 0.8 per cent sodium chloride solutions. It would appear that in the presence of an excess of water in the organism, the binding of some by the glucose may fail to lessen the absolute rate of diuresis. Such a negative effect is illustrated by the following experiment.

EXPERIMENT II.

The Absence of Retardation of Diuresis during Intravenous Injection of Glucose at Low Rates.

The weight of the dog was 14.3 kg. At 8, 9, 10, and 11 a.m. it received by stomach tube 70 cc. of water (total 280 cc.). At 1 p.m. the bladder was emptied by catheter and thereafter every hour. Injection was begun at 2 p.m. In the 1st hour the rate was 0.8, in the second 0.9, in the third 1.0 gm. per kg., glycosuria appearing 10 minutes after beginning the latter rate. The bold-faced figures show hours of injection. The rate of water supply was the same during the preparatory and the injection hours.

Hour.	Urine.	Remarks.
	cc.	
2 p.m.	44	
3 "	39	Injection 0.8 gm. glucose per kg.
4 "	48	" 0.9 " " " "
5 "	52*	" 1.09 " " " "
6 "	48	

* Glycosuria in the 3rd hour.

Both types of result have been observed during intravenous glucose injections in man, but owing to the undesirability of catheterizing normal individuals for a sharp separation of the urine at short intervals, the greater frequency of psychic and nervous influences, and other factors which make it difficult to secure uniform rates of diuresis during the control periods, observations of diuresis in man have been unsatisfactory. (Although Pavy[5] had described retardation of diuresis during low rate intravenous injections of glucose, we had not succeeded in confirming his observation at the time of our preliminary report.)

Injections at Rates between 1 and 2 Gm. of Glucose per Kg. per Hour.

With injections of 1.8 to 2.0 gm. per kg. per hour glycosuria begins promptly and quickly attains a maximum, after which it proceeds at a uniform rate hour after hour for at least 8 hours and probably much longer. Reference to the record of Experiment III, which is typical, will show that the total excretion during the 1st hour may equal that of later hours, indicating the promptness with which the maximum glycosuria may be established with an injection at this low rate.[6]

The absolute rate of the glycosuria may differ in different individuals, and probably in the same individual under different physiological conditions.

In Experiment III the average excretion was 0.42 gm. per kg. per hour, or 2.1 per cent of the intake. Excretions representing

[5] Pavy, F. W., *J. Physiol.*, 1899, xxiv, 479.

[6] With higher rates of injection (2.7 to 7.2 gm. per kg. per hour) the glycosuria gradually accelerates during the first 1 to 5 hours or more before constancy is finally attained, the time required depending largely on the rates of glucose administration and on the water balance.

about 2 per cent of the intake have been seen in other experiments, but one dog receiving 1.8 gm. of glucose per kg. per hour excreted 1.90, 1.75, 2.04, 1.62, 1.61, and 1.97 gm. of glucose per 10 kg. in successive hours, an average of 1.8 gm. per hour representing 10 per cent of the injection.

The glycosuria has been observed to cease within 6 minutes after stopping the injection; *i.e.*, if the bladder is washed out immediately after ending the injection, the next urine collected has been found free of sugar.

The Behavior of Water during Injections at Rates of 1.8 to 2.0 Gm. per Kg. per Hour.

1. Within rather wide limits the quantity of water given before an experiment or during the glucose injection may be varied without influencing the rate of glucose excretion. This is typically illustrated in the following experiment.

EXPERIMENT III.

The Constant Supply and Constant Excretion of Glucose While the Supply and Excretion of Water Varies.

The subject was a bull terrier weighing 16.7 kg., which had served in four previous experiments of similar character. Two pumps, with the delivery tubes anastomosed, discharged water and 37.01 per cent glucose solution respectively through a single cannula. The pump discharging glucose solution ran at a constant rate to deliver 53.9 cc. per 10 kg. of body weight per hour (containing 20 gm. of glucose and 42.6 cc. of water). The second pump delivered water at rates which were varied between 53.9 and 107.8 cc. per kg. per hour. The rates of injection and elimination of glucose and water per 10 kg. of body weight are tabulated by hours from the beginning of the injection.

Injected.			Excreted.		
Hour.	Glucose.	Water.	Glucose.	Urine.	Urinary glucose concentration.
	gm.	*cc.*	*gm.*	*cc.*	*per cent*
1	20	96.5	0.43	16.8	2.57
2	20	96.5	0.31*	6.2	4.90
3	20	151.5	0.52*	59.8	0.87
4	20	123.5	0.44	118.3	0.37
5	20	96.5	0.42	128.0	0.35
6	20	96.5	0.44	82.5	0.59
7	20	96.5	0.41	58.5	0.62
8	20	96.5	0.42	67.0	0.58

* Average 0.42 gm.

In this experiment it will be noted that in the 2nd hour when the urine was only 6.2 cc. and contained nearly 5 per cent of glucose, the excretion was 0.1 gm. below the average of the 1st hour and the last 5 hours, whereas in the 3rd hour when the urine rose to 58.9 cc. it was a trifle above. But the average of the 2nd and 3rd hours was 0.42 gm., the same as for the others, and this fluctuation would be accounted for if only a 2 cc. residuum of the concentrated urine had remained in the bladder, urinary passages, and kidneys to be flushed out during the diuresis of the following hour. There might of course be some retention in the tissues also if the supply of water was too small. In the 7th hour, with the same urinary volume as in the 3rd, the excretion of glucose was 0.41 gm. These observations parallel those made with injections at the 0.8 to 0.9 gm. rates and imply that the glucose utilization rate, as well as the excretion rate, may remain the same during marked fluctuations of the water balance.[7]

2. Unlike injections at subtolerant rates, those productive of glycosuria, may, as is well known, cause acceleration of diuresis. But as in the case of the former, the more rapid injections may be attended by no absolute change of the rate of diuresis, or they may lead to absolute retardations. Which effect will occur appears to depend wholly on the supply of available water in the organism and on the rate of the glycosuria, other factors remaining constant. In experiments with injections at these rates it is possible, under suitable conditions, to demonstrate diminished diuresis during the injection of 1.8 to 2.0 gm. of glucose per kg., followed by sudden liberation of water into the urine when the injection is stopped, exactly as in the case of subtolerant injections; only to do so the water balance must be kept at a relatively lower level.

Frequently in the course of clinical work with 18 per cent glu-

[7] With higher rates of glucose injection, and with wider variation of the water balance at the present rates, the excretion may be affected. However, these observations would not be in harmony with A. A. Epstein's statement (Studies on Hyperglycemia in Relation to Glycosuria, New York, 1916, 69) that "diuresis in diabetes plays an important rôle in determining the total amount of sugar eliminated in the urine, but has no influence on its concentration." They agree, on the other hand, with the finding of T. Addis and C. K. Watanabe (*J. Biol. Chem.*, 1917, xxix, 404) that "changes in the volume of the urine or in the urea concentration of the urine have no appreciable effect on the rate of urea excretion."

cose solutions, an overstepping of the tolerance has been attended by increased diuresis. With the same solution in dogs, decreased diuresis has at times been observed even when a considerable glycosuria has been in progress, as illustrated in the 2nd hour of Experiment III, but the effect is better shown by experiments in which solutions of 50 per cent concentration or above have been used, as in the following.

EXPERIMENT IV.

Retardation of Diuresis during Intravenous Injection at a Rate Productive of Glycosuria, with Acceleration of Diuresis after Injection.

The dog weighed 17 kg. During the 12 hours preceding the experiment it received no food, but water was provided in the cage. At 8.30 a.m. 55 cc. of water were given by stomach tube and the dog was placed on the animal board and prepared for intravenous injection. At 9.30 it was catheterized and thereafter the urine was drawn by catheter every hour. Intravenous injection was begun with 53 per cent solution at the rate of 1.7 gm. of glucose per kg. per hour; *i.e.*, 55.1 cc. of the solution per hour. The injection was continued for the next 4 hours, ending at 4.30 p.m. At 1.30 the urine contained sugar. At 4.30 it contained 4.4 per cent (total 0.35 gm., representing 2 per cent of the intake).

Hour.	Urine volume.	Remarks.
	cc.	
10.30	32	
11.30	15	
12.30	**15**	Injection started.
1.30	**10**	
2.30	**8**	
3.30	**13**	
4.30	**8**	Injection ended.
5.30	65	
6.30	98	
7.30	20	

During the following 12 hours the average urination was 18 cc. per hour.

In this experiment the retardation of diuresis during the injection is comparable to that seen with injections at subtolerant rates. The high diuresis following the discontinuance of injection is even more pronounced and of longer duration.

The highest concentration of glucose which has been observed in the urine during the present experiments with injections at rates below 2 gm. per kg. per hour has been 8.5 per cent.

It may be stated in anticipation of later papers that the urinary glucose concentration, even with injections at rates as high as 7.2 gm. per kg. per hour, has not been driven higher than 8 to 9 per cent except in the case of a few animals. Since in pancreatic and phlorhizin diabetes concentrations of 12 to 13 per cent are easily produced, the point may have theoretical significance.

DISCUSSION.

The Maximum Rate at Which Glucose Can Be Administered Continuously via the Systemic Blood Stream and Fail to Cause Glycosuria.

The value of 0.85 gm. per kg. per hour agrees only approximately with the findings of Blumenthal[8] and others who have used his method (Comessatti,[9] and Loeb and Stadler[10]). Blumenthal made repeated single injections into rabbits with a hand syringe at 15 minute intervals, each injection consuming 10 to 15 seconds during total periods as long as 3 hours. The tolerance limit in dogs (originally expressed in gm. per kg. per $\frac{1}{4}$ hour, and here recalculated for the hour basis) was found as low as 0.6 and as high as 1.3 gm. per kg. The present experiments lasting as long as 7 hours with consistent results varying with few exceptions only between 0.8 and 0.9 gm. define the normal limit more sharply.

The Significance of the Observation That, within Limits, the Quantity of Water Administered before or during Sustained Glucose Injections at Fixed Rates Need Not Affect the Rate of Glucose Utilization or Elimination.

Whatever variations of the blood and tissue volumes and of the blood and urinary sugar concentrations were involved in the fluctuations of the water balance, they did not noticeably affect

[8] Blumenthal, F., *Beitr. chem. Phys. u. Path.*, 1905, vi, 329.

[9] Comessatti, G., *Beitr. chem. Phys. u. Path.*, 1907, ix, 67.

[10] Loeb, O., and Stadler, H., *Arch. exp. Path. u. Pharm.*, 1914, lxxvii, 326.

the rate of glucose metabolism or excretion as long as the rate of glucose supply was constant. Epstein,[11] working with diabetics, also found that the quantity of glucose excreted might be the same in cases showing different blood sugar concentrations and *vice versa*. Such an effect is strikingly illustrated by an unpublished experiment of our own in which a dog received glucose by vein at the rate of 36 gm., and then 27 gm. per 10 kg. per hour, while the water administration was voluntarily varied with a second pump. In the last hour of the faster glucose injection the excretion was 15.2 gm.; in the 1st hour of the reduced rate of injection, it was 10.23 gm., but the blood sugar concentration in both hours was 0.89 per cent. The occurrence of such phenomena is of course tacitly implied in Ambard's attempts to formulate laws governing them. They are also in keeping with the older observation that the drinking of water and passage of urine need not affect the protein metabolism or the total heat production of the organism, as illustrated specifically by a calorimeter experiment of Lusk's[12] in which an 8.8 kg. dog received 200 cc. of water by stomach with no influence on the metabolism. *But the question arises as to how water may dispose itself in the organism to permit such results and how their recognition bears on current conceptions of the significance of measurements of the concentration of various substances in the blood.*

It is clear that the rate of supply to a given organism at a given time is the chief factor which determines the velocity of chemical change and the rate of elimination, but the mechanism is less obvious. Variations of the total quantity of a glucose solution of fixed concentration on one side of an ordinary dialyzer would not vary the rate at which glucose molecules diffused through it, nor could the concentration be changed *without* affecting the rate of diffusion. But with the kidney membrane and cells in general it is not merely the concentration which determines the rates of excretion and utilization respectively. This would suggest that the surfaces of contact between the plasma and the cells must vary in a definite manner with the volume and glucose concentration of the plasma.

[11] Epstein;[7] see also *Proc. Soc. Exp. Biol. and Med.*, 1916, xiii, 67, and Epstein, A. A., and Baehr, G., *J. Biol. Chem.*, 1914, xviii, 21.

[12] Lusk, G., *J. Biol. Chem.*, 1912–13, xiii, 41 and 45.

The following, in which cells and plasma represent phases of a system, serves for illustration. If a single spherical cell such as a lymphocyte is suspended in plasma containing glucose, then (when a state of equilibrium has been approached) the velocity at which glucose molecules strike and penetrate its surface, and therefore the velocity at which they react chemically with substances in the cell interior, will, other factors remaining the same, be determined by the concentration of glucose in the plasma and the area of the surface of contact between the cell and the plasma. If the concentration of glucose in the plasma were decreased, by increasing the volume through the addition of water, and *if the cell surface remained unchanged*, this would lessen the frequency with which glucose molecules impinged on the cell, and entail a proportional decrease of the number entering in a unit of time, etc. In like manner, abstraction of water from the plasma with a decrease of its volume and increase of the glucose concentration would increase the rate at which glucose molecules entered the cells and reacted within it. In either of these hypothetical cases the volume change in the plasma would involve changes in the rate of the glucose metabolism of the cell, contrary to the experimental findings under discussion. If, however, as the plasma volume increased or diminished (and the glucose concentration varied correspondingly) the surface of contact between the cell and the plasma increased or diminished in the same proportion, then the total number of glucose molecules impinging on the total cell surface would theoretically remain the same and such an occurrence would explain the experimental findings.

The necessary condition would be fulfilled if while the total content of glucose in the plasma remained fixed and the volume of plasma varied, the ratio of the plasma volume to the surface of contact between the plasma and the cell remained constant; that is, in the body taken as a whole, if *the surface of contact between the plasma and cell phases varied directly as the plasma volume or inversely as the plasma glucose concentration.*

These observations are in harmony with those of Epstein concerning the ratio of the plasma volume to the blood cell volume, the blood sugar concentration, and the rate of glycosuria in diabetics, which led to the conclusion that the total sugar content of the blood fixes the rate of glycosuria. Other things being equal,

it should do so in accordance with the above, *as long as the specific surface of the plasma remains fixed.*

As to how the surface of contact between the plasma and the cells of the body may be regulated to meet changing volumes of blood, several ways are apparent. In the histological sense, the single leukocyte suspended in plasma may increase in volume as water is added to the plasma, or diminish when glucose is added. Dilution of the plasma may separate cells in contact, concentration permit separate cells to cluster together. The wall of the capillary may be more or less distended, the glomerular tuft may inflate and deflate, providing more or less surface in proportion to the blood volume. Or the question may be considered in terms of phases in colloid systems, and the influence of varying contents of sugar and water on their volumes and specific surfaces. As Henderson[13] recalls, Willard Gibbs proved that when equilibrium is established the state of the system is unaffected by the volume of the phases.

Acceleration and Retardation of Diuresis Resulting from Intravenous Injections of Glucose.

The phenomenon of decreased diuresis during injection, with a sudden liberation of water into the urine when the injection is ended, is quite analogous to that observed as a result of alimentary glucose administration by Lusk[12] and Fisher and Wishart.[14] The analogy is made striking by a recalculation of their data, which shows that glucose actually entered the blood by absorption during periods of 2 to 5 hours at mean rates of 1.6 to 1.7 gm. per kg. per hour, as compared with direct injections of 0.8, 1.8, and 2.0 gm. per kg. per hour in the present series.

Fisher and Wishart administered 50 gm. doses of glucose by stomach to dogs weighing 8 to 9 kg., sacrificed them at hour intervals, and found that absorption was completed "during the 4th hour." If the absorption time was 3.5 hours and the average body weight 8.5 kg., this would imply a mean absorption rate of 1.68 gm. per kg. per hour. Lusk found that 50 gm. of glucose might cause a 20 per cent increase in metabolism ending "*in the 4th hour,*" and 75 gm. might also cause a 20 per cent increase

[13] Henderson, L. J., *Proc. Nat. Acad. Sc.*, 1916, ii, 654.

[14] Fisher, G., and Wishart, M. B., *J. Biol. Chem.*, 1912–13, xiii, 49.

which continued "*through the 5th hour.*" As Lusk states, Fisher and Wishart showed "that there is a large retention of water by the organism during the period of high metabolism, which water is suddenly eliminated during the last hour of the high metabolism." In the case of the 50 gm. doses the period of high metabolism ended in the same hour that absorption was completed. From the present experiments it is apparent also that the period of high diuresis comes promptly when the flow of glucose into the blood is ended. Therefore, no doubt, as Lusk assumed, the end of the high metabolism and the onset of high diuresis following the administration of 75 gm. of glucose also marked the end of absorption as directly observed in the case of the smaller doses. Then taking the dog's weight at 7.3 kg.[15] and the time as 5 hours, the rate of absorption would have been 1.6 gm. per kg. per hour as against 1.7 gm. reckoned in the case of the 50 gm. doses. The fact that the metabolism was increased to the same level with the 50 and 75 gm. doses in the same animal confirms the reckoning which shows that the same absorption rate prevailed in the two cases.

Interpretation of the Effects of Glucose on the Water Balance.

The retention of water was attributed by Lusk and by Fisher and Wishart to the increased osmotic power of the organism due to the presence of increased amounts of freely diffusible sugar. Following the alimentary administration of 50 gm. of glucose in 150 cc. of water they found during the 1st hour a rapid absorption of glucose into the blood but no fall of the hemoglobin percentage to indicate hydremia. It was presumed that in this hour the balance of glucose still in the alimentary tract held water back. But by the end of the 2nd hour, two-thirds to three-quarters of the glucose having been absorbed, they found evidences of hydremia, and in the 4th hour (absorption completed) hydremia passed and hydruria came. Taken in conjunction with well known observations that direct intravenous injection of glucose may lead promptly to hydremic plethora and that glucose so injected passes with great rapidity from the blood into the tissues where it promptly ceases to exist as such, this presents the picture of a tide of glucose and water molecules moving together through the organism, the glucose finally disappearing within the cells and the water into the urine.

At the present time it might seem preferable to express the matter in terms other than those of the osmotic theory. Other-

[15] The experiment of March 27, 1912, Lusk,[12] pp. 44–45.

wise the above conception appears adequate to explain the variations of the water balance seen in connection with intravenous injections of glucose. In accordance with the solvate theory,[4] glucose molecules would tend to hydrate themselves, the number of water molecules held by a molecule of glucose depending upon the individual hydration capacity of glucose, on the amount of water present, and on the amounts and individual hydration capacities of other molecules in the solution, etc. During intravenous injections at subtolerant rates when the preponderance of all the free glucose in the body is in the blood, its hydration should lead to hydremia and this to decreased diuresis, unless an excess of water is injected with the glucose. But when glucose is injected faster than it can be chemically changed in the cells, and so passes through the renal cells and appears on the urinary side of this membrane, water must tend to accumulate there also; how much, should depend upon the quantitative distribution of glucose between the blood and urine phases, on the total supply of water in the body, etc.

This would imply that the diuretic effects of glucose are an expression of the power of the glucose molecule to hydrate itself, and of the presence of hydrated glucose on the urinary side of the renal cell. Hofmeister[16] attributed the power of salts to dehydrate colloid gels to the tendency of the salt to hydrate itself. Fischer[17] has drawn a parallel between the diuretic effects of salts and their power to dehydrate protein gels, and made experiments tending to show that the parallel holds for sugar. The above interpretation is therefore in harmony with the view previously expressed, although differing in detail.

The present results and interpretation are contrary to the generality of Allen,[18] that all the common sugars are diuretics when given intravenously, that the phenomenon of water retention during glucose administration is observed only when the sugar is given by the alimentary, subcutaneous, or other indirect route, and contrary also to his conception that antidiuresis is dependent upon the union of glucose with a colloidal substance, etc.

[16] Hofmeister, F., *Arch. exp. Path. u. Pharm.*, 1890, xxvii, 395.

[17] Fischer, M. H., Edema and Nephritis, New York, 2nd edition, 1915.

[18] Allen, F. M., Studies on Glycosuria and Diabetes, Cambridge, 1913, 1052–4.

SUMMARY AND CONCLUSIONS.

1. The technique employed in making continued injections of glucose and water into the veins of animals (rabbits, dogs) at known rates by means of a previously described volumetric pump is reported in detail, with a note on the purification of commercial glucose.

2. Experiments are cited to show that the maximum rate at which glucose can be administered continuously by vein to normal resting rabbits and dogs without causing gross glycosuria (*i.e.*, the normal intravenous glucose tolerance limit) is close to 0.85 gm. per kg. per hour in the great majority of cases, but may be found, more frequently than in man, a little higher (0.9 gm. per kg. per hour). Within rather wide limits the water administered simultaneously with the glucose may be varied without appreciably changing the rate of glucose utilization.

3. Continued intravenous injections of glucose at uniform rates between 0.9 and 2.0 gm. per kg. per hour may lead to continued excretions of glucose in the urine at uniform rates. The ratio of the intake to the output rate differs with different individuals receiving glucose at the same rate, and with different rates of injection in the same individual. When the injection rate is 1.8 to 2.0 gm. per kg. per hour, the glucose excretion per hour has represented as little as 2 and in one case as much as 10 per cent of the injection. Within rather wide limits variation of the quantity of water injected and eliminated during constant glucose injections at these rates has not been found to affect the rate of glucose utilization or excretion.

4. For any individual case the rate at which glucose enters the blood determines the rate of glucose utilization and excretion regardless of the volume of the blood and urine or the concentration of glucose in them.

5. The observed constancy of the velocities of glucose metabolism and excretion, in spite of such changes, is explained on the basis that water must so distribute itself in the body that the ratio of the plasma volume to its surface of contact with the cells at large (specific surface of the plasma phase) remains constant. The terms plasma and cells are employed in the general sense of phases in heterogeneous systems.

6. By controlling the rates of glucose and water injection it has been found possible to produce relative and absolute retardations of diuresis during glucose injections at rates causing glycosuria, as well as with those at subtolerant rates. But acceleration of the rates of water elimination (over and above that ascribable to the water given with the glucose) has not been observed without glycosuria.

7. The tendency toward water retention is attributed to the presence of free diffusible glucose in the blood and, to a less extent, in the tissues and the tendency of the glucose to hydrate itself in accordance with the solvate theory. The tendency toward diuresis, manifest only when glucose gains access to the urine, is regarded as an expression of the same process operative in another place. Where the balance of water will be found in the body during injections of glucose will depend at any moment on the quantitative distribution of glucose among the phases of the organism. Increased diuresis occurs when there is glycosuria and a sufficient supply of water in the organism.

8. Attention is called to the probable fallacy of considering the concentration of any substance in the blood plasma as a reliable index *per se* of the rate at which it enters the cells, of the rate of its utilization or excretion, or of the toxic effects produced.

PEPTONE HYPOGLYCEMIA.

By HUGH McGUIGAN and E. L. ROSS.

(*From the Laboratory of Pharmacology, Northwestern University Medical School, Chicago.*)

(Received for publication, April 20, 1917.)

In 1915 we reported work[1] showing that the intravenous injection of peptone in dogs may cause a marked decrease in the concentration of sugar in the blood, a condition which we desig-

CORRECTION.

On page 42, Vol. XXX, No. 1, May, 1917, Experiment 3 should read "*with injection of 0.3 gm.*" instead of *3.0 gm.*

sugar. The action of animal products, however, [illegible] function is so contradictory that this observation cannot for the present be definitely accepted or evaluated. The subject is discussed by Allen.[4]

The work of Kuriyama on hypoglycemia has no direct relation to ours, and cannot be used to refute our results because he obtained little or no peptone reaction. To use his own words:

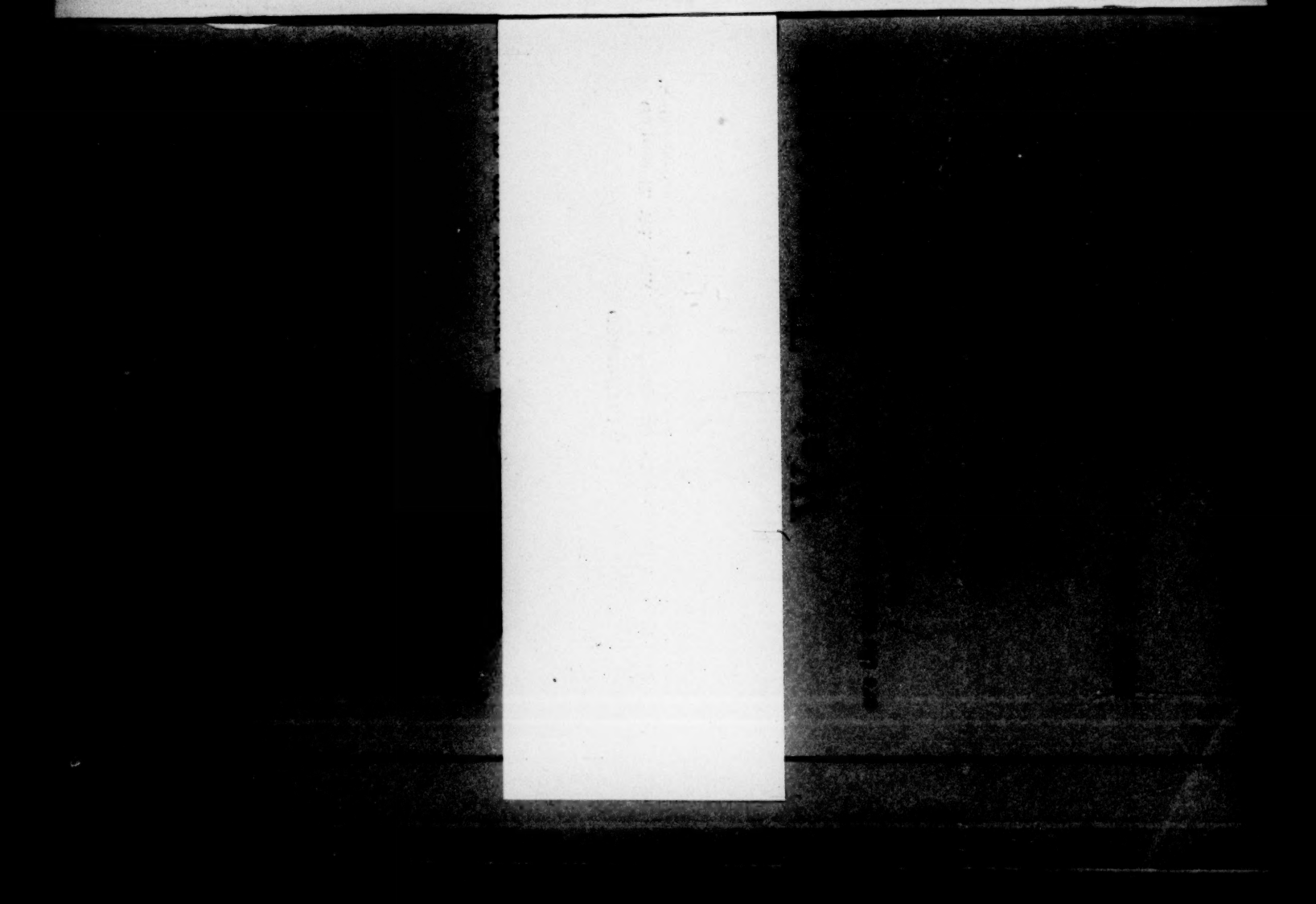
"Full grown rabbits were used. . . . . The rabbit is usually considered to be less susceptible to peptone. . . . . In my own experi-

[1] McGuigan, H., and Ross, E. L., *J. Biol. Chem.*, 1915, xxii, 417.

[2] Kuriyama, S., *J. Biol. Chem.*, 1917, xxix, 127.

[3] Glaessner, K., and Pick, E. P., *Z. exp. Path. u. Ther.*, 1909, vi, 313.

[4] Allen, F. M., Glycosuria and Diabetes, Cambridge, 1913, 526, 555, and ff.



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In 1915 we reported work[1] showing that the intravenous injection of peptone in dogs may cause a marked decrease in the concentration of sugar in the blood, a condition which we designated as peptone hypoglycemia. The conclusions of this work were opposed to the statement of Henderson and Underhill that the intravenous injection of peptone in dogs causes glycosuria. In a recent article Kuriyama,[2] working under Underhill's direction, but using rabbits instead of dogs, confirms our experiments to the extent that he could find no glycosuria, but he failed to find evidence of hypoglycemia.

The work of Glaessner and Pick[3] is in harmony with ours. They found that peptone inhibits epinephrine glycosuria. Pancreatic juice has a similar action. Kuriyama and Underhill, however, explain this as due to decreased permeability of the kidney for sugar. The action of animal products, however, on kidney function is so contradictory that this observation cannot for the present be definitely accepted or evaluated. The subject is discussed by Allen.[4]

The work of Kuriyama on hypoglycemia has no direct relation to ours, and cannot be used to refute our results because he obtained little or no peptone reaction. To use his own words:

"Full grown rabbits were used. . . . . The rabbit is usually considered to be less susceptible to peptone. . . . . In my own experi-

[1] McGuigan, H., and Ross, E. L., *J. Biol. Chem.*, 1915, xxii, 417.

[2] Kuriyama, S., *J. Biol. Chem.*, 1917, xxix, 127.

[3] Glaessner, K., and Pick, E. P., *Z. exp. Path. u. Ther.*, 1909, vi, 313.

[4] Allen, F. M., Glycosuria and Diabetes, Cambridge, 1913, 526, 555, and ff.

ments the rabbits usually showed no severe symptoms except a slight degree of prostration after an intravenous injection of Witte's peptone in doses of 0.5 to 0.75 gm. per kilo of body weight."

In the animals used by us there was always a pronounced and exhausting effect, with vomiting and diarrhea. In describing the conditions that produce hypoglycemia we emphasized fatigue and exhaustion as important causative factors, and while we cannot yet explain the mechanism of the action, we still hold, as then, that it may be a condensed fatigue effect. We say condensed, because most conditions, like fatigue or moribund states, that produce hypoglycemia, do so not acutely but only in the prolonged cases. It is evident therefore that we should not expect hypoglycemia from peptone in those animals, like rabbits, in which it does not act. Many drugs, also, *e.g.*, atropin, which produce marked changes in the dog, may be almost without action in the rabbit.

We have again tested this action of peptone in dogs, and determined the sugar by two methods of analysis. The following tables show the results.

EXPERIMENTAL.

The animals used in the following tests were not uniform in any way. Some were not fed for 18 hours before the peptone was injected while others had considerable food in their stomachs at the time of the experiment. Most of the animals were in good condition. A few were suffering from distemper. The diet of the dogs was mixed and variable, being the scraps from the hospital dining-room tables.

The blood sugar was determined by a modification of Bertrand's and also by Benedict's method. Triplicate determinations were made by each method.

The Bertrand method was carried out as follows. 10 cc. of oxalated blood were measured into a beaker containing 30 cc. of distilled water. After laking, enough picric acid was added to precipitate the proteins, then the volume of the solution was made up to 150 cc. with saturated picric acid solution. The mixture was filtered and 100 cc. of the filtrate without removal of the picric acid were taken for the determination. This sample was brought to a boil and to it were added 60 cc. of freshly prepared boiling Fehling's solution. The mixture was kept boiling for 2 minutes.

It was then filtered through asbestos, washed, and the reduced copper dissolved in 25 cc. of 5 per cent ferric sulfate in 20 per cent H_2SO_4 solution, and titrated against standard permanganate solution of such strength that 1 cc. of $KMnO_4$ represented 0.001 gm. of dextrose. The Fehling's solution used was that made up according to U. S. P. No. IX.

The Benedict method was carried out as described by Myers and Bailey.[5]

The peptone injected was weighed out in varying quantities, from about 0.3 to 0.5 gm. per kilo of body weight. 10 to 15 cc. of physiological salt solution per gm. of peptone were used. The solution was filtered through gauze but not boiled before being injected into the animal.

The animal was tied on a dog board and bled with a hypodermic syringe from the jugular vein. Immediately after the bleeding, the peptone was injected, sometimes into the jugular vein and sometimes into the femoral vein.

TABLE I.

Blood Sugar Changes Caused by Intravenous Peptone Injections.

Dog.		Peptone.	Before peptone.		2 hrs. after peptone.		4 hrs. after peptone.	
No.	Weight.		Bertrand method.	Benedict method.	Bertrand method.	Benedict method.	Bertrand method	Benedict method.
	kg.	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	39.0	10.0	0.096	0.110	0.105	0.116	0.067	0.076
2	25.0	6.5	0.097	0.097	0.021	0.040		
3	15.5	3.5	0.125	0.119	0.108	0.112	0.090	0.096
4	15.0	4.0	0.087	0.088	0.060	0.062	0.076	0.061
5	12.0	3.0	0.090	0.091	0.052	0.055	0.075	0.081
6	35.0	8.5	0.066	0.065	0.020	0.037		
7	19.0	4.5	0.105	0.103	0.138	0.156	0.036	0.060
8	33.0	8.0	0.070	0.098	0.060	0.098	0.060	0.069
9	35.0	8.0	0.068	0.087	0.048	0.051		
10	29.0	7.0	0.058	0.088	0.072	0.109	0.047	0.060
11	26.0	3.5	0.078	0.100	0.048	0.080	0.054	0.079
12*	30.0	4.5	0.090	0.093			0.068	0.069
13*	26.0	2.9	0.085	0.105			0.063	0.069

* Fehling's solution used in these cases contained 3 per cent KOH.

[5] Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.

TABLE II.

Fall in Blood Sugar Caused by Intravenous Peptone Injections.

Dog.	Before peptone.		After peptone.			Fall in glycemia.	
	Bertrand method.	Benedict method.	Time after injection.	Bertrand method.	Benedict method.	Bertrand method.	Benedict method.
			hrs.				
1	0.096	0.110	4	0.067	0.076	0.029	0.034
2	0.097	0.097	2	0.021	0.040	0.076	0.057
3	0.125	0.119	4	0.090	0.096	0.035	0.023
4	0.087	0.088	2	0.060	0.062	0.027	0.026
5	0.090	0.091	2	0.052	0.055	0.038	0.036
6	0.066	0.065	2	0.020	0.037	0.046	0.028
7	0.105	0.103	4	0.036	0.060	0.069	0.043
8	0.070	0.098	4	0.060	0.069	0.010	0.029
9	0.068	0.087	2	0.048	0.051	0.020	0.036
10	0.058	0.088	4	0.047	0.060	0.011	0.028
11	0.078	0.100	4	0.054	0.079	0.024	0.021
12	0.090	0.093	4	0.068	0.069	0.022	0.024
13	0.085	0.107	4	0.063	0.069	0.022	0.036
Average.....	0.0858	0.0957		0.0528	0.0633	0.0330 38 per cent	0.0324 34 per cent

RESULTS AND DISCUSSION.

From the results of the work as given in Table I it is clear that in every case within 4 hours after the injection of peptone there was a fall in the blood sugar as determined by both methods. In three dogs there was a rise in the blood sugar preceding the fall. The same was the case with at least four dogs reported in our previous paper.[1] We believe that if the blood were examined at short intervals a slight rise preceding the fall in blood sugar would always be found. A greater fall might also be found in many cases, if the time of analysis were extended.

The results by the two methods, while in the same direction, seldom agree as closely as the duplicates of the same method. The Benedict method usually gives higher results. In Table II where the blood sugar before peptone and the minimum blood

sugar after peptone are compared, the differences between the determinations by the two methods are best shown. In the averages before peptone the Benedict method gives 0.0099 per cent higher than the Bertrand method and in the averages of the minimum blood sugar after peptone the Benedict method gives 0.0105 per cent higher than the Bertrand method. Both are practically 0.01 per cent higher. Further work on the differences between the methods is being carried out. We can say positively, however, that variations in the sugar level in dogs as reported by different investigators are due mainly to the concentration of the alkali in the Fehling's solution which they used. The term Fehling's solution in the literature means anywhere from 5 per cent to 12.5 per cent KOH in the mixed fluid. While in a water solution of dextrose a variation of the alkali causes a relatively slight change in the sugar found, in a blood solution it makes a remarkable difference. Because of this hitherto unappreciated factor the level of the blood sugar has been placed anywhere from 0.03 to 0.150 per cent. For this reason in our previous paper we think our results are relatively low. The general conclusions, however, are correct.

In Table II the figures show clearly that there is always a hypoglycemia after the injection of peptone. Sometimes it comes earlier than in others. The cause of this we cannot say with certainty. We believe that some animals under certain conditions of health and feeding become fatigued more rapidly than others and the fatigue is the main cause of the hypoglycemia. The average fall in glycemia amounted to 0.03 per cent, which constituted 34 to 38 per cent of their normal blood sugar.

SUMMARY AND CONCLUSIONS.

Dogs, not uniformly controlled as to diet, general condition, or time after eating, were injected intravenously with 0.3 to 0.5 gm. of Witte's peptone per kilo of body weight. The blood sugar was determined before the injection and at intervals of 2 and 4 hours after the injection. In every case hypoglycemia developed. The average fall for thirteen dogs was 0.03 per cent dextrose or 34 to 38 per cent of their original blood sugar.

THE RÔLE OF YEAST IN THE NUTRITION OF AN INSECT (DROSOPHILA).

By J. H. NORTHROP.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, April 21, 1917.)

It has been found by several authors[1] that the fruit fly, *Drosophila ampelophila*, when freed from microorganisms and raised under aseptic conditions grows much more normally on fruit or potato when yeast is present. This applies strictly, however, only to the growth of the larvæ, it having been found[2] that the imago is able to live the normal length of time on food which contains no yeast and which is inadequate for the growth of the larvæ. This is in accord with the results of McCollum[3] and Osborne and Mendel,[4] who have found that food which is insufficient for the growth of rats may be sufficient for their maintenance. It is well known from the work of Funk,[5] Hopkins,[6] Osborne and Mendel,[7] McCollum,[8] and others, that certain accessory food substances are required for the growth of rats, pigeons, chickens, and probably the higher animals in general. It has further been shown that yeast is especially rich in these substances. It seemed of importance, therefore, to determine whether in the case of *Drosophila* the yeast was needed to supply some accessory food substance or whether the insects

[1] Guyénot, E., *Compt. rend. Soc. biol.*, 1913, lxxiv, 178, 223. Loeb, J., and Northrop, J. H., *J. Biol. Chem.*, 1916, xxvii, 309.

[2] Loeb and Northrop, *Proc. Nat. Acad. Sc.*, 1917, iii, 382.

[3] McCollum, E. V., *Am. J. Physiol.*, 1911–12, xxix, 215.

[4] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xxiii, 439.

[5] Funk, C., Die Vitamine, Wiesbaden, 1914. See also *J. Physiol.*, 1912–17, and *J. Biol. Chem.*, 1913–17.

[6] Hopkins, F. G., *J. Physiol.*, 1912, xliv, 425.

[7] Osborne and Mendel, *Carnegie Institution of Washington, Publication 156*, 1911, pts. i and ii, and *J. Biol. Chem.*, 1912–17.

[8] McCollum and collaborators, *J. Biol. Chem.*, 1913–17.

lived exclusively on yeast. Since it is impossible to conduct direct metabolism experiments on a single insect it was necessary to use indirect methods. This has been done in two ways; (1) by determining the number of flies that may be raised on a definite mixture of yeast and banana or yeast and casein, and (2) by determining the rate of growth of the larvæ on such mixtures.

1. In the first series of experiments 0.025 gm. of banana was weighed into each of fifteen test-tubes. Similar series were prepared containing 0.025 gm. of (*a*) casein, (*b*) sugar, and (*c*) 0.025 gm. of each. 0.25 cc. of a suspension of yeast in water containing 10 gm. of yeast[9] in 100 cc. was added to each tube, the contents were thoroughly mixed, and sufficient absorbent cotton was added to absorb the excess water and prevent the flies from drowning. The tubes were then plugged with cotton and sterilized in the autoclave for 20 minutes. A similar series was prepared in which 0.25 cc. of water was added to the banana, etc., and also one containing the yeast suspension alone. Five to ten pairs of flies were then put in each tube (using the technique of Delcourt and Guyénot[10]) and allowed to remain until 50 to 100 eggs had been laid. This required 2 to 3 days. The flies were then removed and the tubes incubated at 26°. The number of flies which developed were counted. At the end of the experiment the tubes were tested for sterility by inoculation into glucose broth, agar, litmus milk, and yeast extract culture media. Only those tubes which remained sterile were considered in recording the results.

The numbers in Table I refer to the maximum number of flies which developed on any one culture of the series.

The results show that the number of flies which are able to develop on a certain quantity of yeast may be greatly increased by the addition of casein, sugar, or banana to the yeast. These substances must therefore be able to serve as food for the larvæ when yeast is also present. The fact that they are of little or no value when not mixed with yeast shows that the latter supplies some substance or substances which are necessary for the growth of the larvæ and which it cannot obtain, in sufficient quantity at least, from banana, casein, or sugar.

The quantitative results may be seen more clearly from the second series of experiments (Table II) in which the time re-

[9] The yeast used in all these experiments was free from starch and was obtained through the courtesy of the Fleischmann Company.

[10] Delcourt, A., and Guyénot, E., *Bull. Sc. France et Belgique*, 1911, xlv, 249.

TABLE I.

Yeast.	Banana.	Casein.	Sugar.	No. of flies.
gm.	*gm.*	*gm.*	*gm.*	
0.025				4
0.025	0.025			9
	0.025			0*
0.025			0.025	7
0.025		0.025		16
0.025		0.025	0.025	24
		0.025	0.025	0**

* If a large number of eggs are allowed to be laid on a considerable quantity of banana a few small sexually sterile flies[11] may be raised. If the banana is removed aseptically from the fresh fruit and kept sterile without heating, slightly better results are obtained, although it has thus far been impossible to raise a second generation even by this method.

** The ash from 0.025 gm. of yeast was added to this culture.

quired for the larvæ to reach the pupal stage is used as the index of the food value of the mixture.

2. In these experiments the cultures were made in 120 cc. Erlenmeyer flasks plugged with cotton and containing 8 gm. of the mixture. The yeast was added in the form of a 10 per cent suspension and thoroughly mixed with the banana. The flies were only allowed to remain in the flasks for 14 hours and were then removed and the cultures kept at 30°.

TABLE II.

Ratio, banana to yeast.	Time from egg to pupæ.	Time as pupæ.
	days	*days*
0	4.12±0.04	3.43±0.05
1	3.72±0.02	3.62±0.07
2	3.60±0.01	3.68±0.04
4	3.74±0.02	3.54±0.05
8	4.33±0.03	3.49±0.05
16	4.75±0.03	3.23±0.10
32	5.33±0.08	3.45±0.15
64	6.68±0.16	Irregular; flies.
128	8.3 ±0.2	Abnormal.
256	9.0 ±0.2	
∞	14–25	

[11] Loeb and Northrop, *J. Biol. Chem.*, 1916, xxvii, 309. See also Guyénot,[1] 271.

The figures given in Table II are the average times required for all the larvæ to reach the pupal or imago stage (generally 100 to 200). It will be seen that the number of days required for the pupal stage to be reached decreases slightly up to a value for the ratio of banana to yeast of about two, and then increases rather rapidly. This seems to indicate that such a mixture allows a slightly more rapid growth of the larvæ than does yeast alone. As the proportion of banana to yeast becomes larger the results become more irregular, as is shown by the increase in the probable error, until when banana alone is used the average is of little significance. This increase is obviously due to the dilution of the yeast by the banana which, when present in excess, behaves as unavailable material. The irregularity in the results with large proportions of banana is partially due to the fact that as the value of the ratio of banana to yeast increases development becomes abnormal.

The pupal period remains the same, within the limits of error, no matter how long the larvæ may require to reach that stage. The larval period may be more than doubled by decreasing the amount of yeast. This has been repeatedly observed and corresponds to Osborne and Mendel's[4] experiments on the retardation of growth of rats by means of incomplete food. It raises the question as to whether this is an actual prolongation of the life of the insect or merely an increase in the larval period with a corresponding decrease in the length of life of the imago. The fact that the pupal period is independent of the larval period would seem to indicate that the length of life of the imago would be independent of the time required to reach that stage in so far as the variation in the duration of the larval stage is due to lack of adequate food. Experiments are under way to determine this point. Since these experiments were started a note has appeared by Osborne, Mendel, and Ferry[12] in which they give similar indirect evidence on this point by means of the fact that the menopause in female rats is delayed by stunting. There appears to be no direct evidence, however, that there is any definite relation between the duration of the sexual life of the organism and the total life of the organism.

[12] Osborne, T. B., Mendel, L. B., and Ferry, E. L., *Science*, 1917, xlv, 294.

The most probable explanation of these results seems to be that the yeast supplies some substance or substances which are necessary for the growth of the organism and which cannot be obtained by it from protein or carbohydrate. The experiments of Loeb[13] show conclusively that it is not a question of inorganic constituents, since this author was able to raise *Drosophila* on yeast growing on Pasteur's solution in which the salt content could be accurately controlled. These experiments also show that the yeast plant is able to synthesize the accessory substance required by the fly from simple chemical compounds of known structure. Nothing definite can be said as yet as regards the nature of the accessory substance or substances. Attempts to concentrate it by various methods of extraction, etc., have not yet led to any definite results.

It is interesting to note in this connection that Wildiers[14] in 1900 came to the conclusion that some accessory substance that he called "*bios*" was needed for the growth of yeast itself but that this substance could not be synthesized by the yeast plant. A somewhat similar condition seems to exist in regard to the slime molds[15] and amebæ[16] which have been found to be dependent on bacteria for normal growth.

Experiments with Animal Tissues.

It seemed possible that some light might be shed on the problem if other substances could be found which were able to furnish the accessory food substances for the fly. Cultures were therefore made of various animal tissues.

The tissues were removed from the animal, ground in a mortar to a thin paste with a small amount of sand and water, the resulting suspension was poured into test-tubes with sufficient absorbent cotton to prevent the flies from drowning, and sterilized, etc., as usual. The tethelin was received in the form of a sterile powder. It was added aseptically to previously sterilized tubes containing 0.5 gm. of banana or yeast.

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[13] Loeb, J., *J. Biol. Chem.*, 1915, xxiii, 431.

[14] Wildiers, E., *La Cellule*, 1901, xviii, 313. See also Amand, A., *ibid.*, 1902, xx, 225; 1904, xxi, 329.

[15] Pinoy, E., *Ann. l'Inst. Pasteur*, 1907, xxi, 622, 686.

[16] Biedermann, W., Winterstein's Handb. vergl. Physiol., Jena, 1911, ii, pt. i, 278.

Liver, pancreas, and kidney from the dog, and liver from the mouse were found to allow normal and perfect development, and they must therefore contain the accessory substances. Spleen, heart muscle, muscle, blood, adrenal, and thyroid from the dog were insufficient, as were muscle, testis, and tumor from the mouse. Thymus from the dog, rabbit, or calf allowed a few imagos to develop, but growth was abnormally slow, the flies were small, and many pupæ failed to hatch.

Dead sterile flies may also serve as normal food for the larvæ. This shows that the accessory substances are not broken down in the body of the fly but are preserved in an active form.

Experiments in which tethelin was added in quantities up to 60 mg. per 0.5 gm. of banana or of yeast also gave negative results; no change was noted in the growth of the larvæ in either case. The results with pancreas[17] and liver[18] agree with those already found for the higher animals. It is possible that the negative results in the case of some of the tissues may be due in part to the sterilization, but it is difficult to see why this should affect one tissue more than another. The larvæ grew normally on any of the tissues when they were infected with bacteria. This may explain the results of Bogdanow,[19] who was unable to raise sterile meat flies (*Calliphora vomitoria*) on sterile meat (presumably muscle tissue). It seems probable that growth would have been normal on liver or pancreas and that the bacteria merely served to furnish the accessory substances which according to the present experiment are not present, in sufficient amount at least, in muscle.

SUMMARY AND CONCLUSIONS.

It has been shown in a previous paper that the fruit fly, *Drosophila*, when freed from microorganisms cannot be raised successfully on sterilized banana or on a mixture of pure proteins, sugars, salts, and fat. It is shown in the present paper that:

1. The number of flies which are able to develop on a definite quantity of yeast may be increased by the addition of banana,

[17] Eddy, W. H., *J. Biol. Chem.*, 1916, xxvii, 113.
[18] Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 340, 401.
[19] Bogdanow, E. A., *Arch. Physiol.*, 1908, Suppl., 173.

casein, or sugar to the yeast. These latter substances can therefore serve as food for the larvæ when supplemented by yeast.

2. The rate of growth of the larvæ is equally, or slightly more rapid on mixtures of banana and yeast containing more than 33 per cent yeast than it is on yeast alone. In mixtures containing less than this amount of yeast growth becomes slower as the amount of yeast is decreased, and finally, when the proportion of yeast is very small, becomes abnormal. Yeast therefore contains a sufficient excess of the necessary (accessory) substances to render available as food approximately twice its weight of banana.

3. Kidney, liver, and pancreas from the dog, liver from the mouse, and the bodies of the flies themselves are an adequate source of food for the larvæ.

4. Sterilized spleen, heart muscle, muscle, blood, adrenal, and thyroid from the dog are not an adequate food for the larvæ. Muscle, testis, and tumor from the mouse are also inadequate. Sterilized thymus from the dog, rabbit, or calf allow a few imagos to develop, but growth is slow and the flies are abnormally small. No effect on the rate of growth could be noted when tethelin was added to the food.

In conclusion the author wishes to acknowledge his indebtedness to Professor Jacques Loeb for many helpful suggestions during the course of the work. He is indebted to Dr. John Auer for the dog tissues, to Dr. Herbert Taylor for the mouse tissues, to Dr. Uhlenhuth for the thymus, and to Dr. T. B. Robertson for the tethelin.

THE INFLUENCE OF PROTEIN INTAKE ON CREATINE EXCRETION IN CHILDREN.

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The phenomenon of creatinuria in children is of particular interest, for the reason that while the excretion of creatine in man occurs in adults only under pathological conditions, it has been shown by Rose[1] that creatine is a constituent of the urine of all children, both normal and pathological, up to the age of puberty.

Shortly after the appearance of Rose's paper his results were confirmed by Folin and Denis.[2] These investigators did not, however, feel justified in accepting the suggestion of Rose regarding the cause of creatinuria in children, and in their paper make the following statement:

> "We are inclined to believe that the creatine in children's urine does not depend as Rose suggests on a peculiar carbohydrate metabolism but that it is due to an excessively high level of protein consumption (in proportion to mass of muscles in the body). . . . . If the above hypothesis is correct it should be possible to reproduce in adults by forced feeding with protein which contains no creatine the condition with reference to creatine found in children and it should also be possible to obtain creatine-free urine from children by reducing their protein consumption."

In a recent paper it has been shown by one of us[3] that in adults suffering from hyperthyroid disease it is possible by forced protein feeding to obtain urines rich in creatine, and by diminishing the protein intake to a minimum, to obtain from the same

[1] Rose, W. C., *J. Biol. Chem.*, 1911–12, x, 265.

[2] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 253.

[3] Denis, W., *J. Biol. Chem.*, 1917, xxx, 47.

subjects creatine-free urines. In the present paper we wish to present certain experimental results which afford further verification of the suggestion made 5 years ago by Folin and Denis. In short, we have found that by feeding children diets extremely low in protein but of sufficient calorific value, it is possible to obtain urines absolutely creatine-free. On feeding increasing amounts of protein, creatine excretion begins, increases day by day, and if the protein intake can be pushed to a sufficiently high point may become considerably greater than the excretion of preformed creatinine. The experimental method pursued in all cases was first to give a creatine-free diet containing the largest amount of protein the subject could be induced to take. This was followed by a diet containing a minimum amount of protein, which was succeeded by a second period of high protein feeding.

The same articles of food were used for all the children, with slight quantitative changes to suit individual taste.

During the high protein period the food consisted of eggs, cheese, peanut butter, gelatin, milk, bread, potato, and orange juice. During the low protein periods the diet was made up of rice, potato, tapioca, apples, oranges, spinach, 40 per cent cream, butter, sugar, lactose, honey, and arrowroot biscuit (made from arrowroot starch and butter).

In the case of the infant a mixture of whole and fat-free milk was used during the high protein period, while for the low protein diet we used a mixture of oatmeal water, lactose, and 40 per cent cream. Analysis made of these formulas indicated that during the period of high protein feeding this infant was receiving daily 53 gm. of protein, 28 gm. of fat, and 75 gm. of carbohydrate, while during the period of low feeding he was given 7.8 gm. of protein, 41 gm. of fat, and 105 gm. of carbohydrate. The calorific values of the two diets were approximately equal, being equivalent to about 90 calories per kilo of body weight of the infant.

Of the four children used three were very active and were up and about the ward daily; the fourth (Subject III) had a cast on one leg and was therefore obliged to remain in bed. The infant was kept on a Bradford frame throughout the experimental period in order to facilitate the collections of urine.

Body temperatures were taken twice daily but as none above normal were recorded, no detailed record of this has been included in the tables.

Creatinine and creatine were determined by Folin's micro methods.[4] For the determinations of total nitrogen the method of Folin and Denis[5,6] was employed. The subjects of our experiments were all patients in the children's ward of this hospital. Two may be considered as essentially normal (Subjects IV and V); two were in a late stage of convalescence (Subjects II and III). All remained in good condition and maintained their weight during the experimental period.

Subject I (*Table I*).—A girl, 5 years old, weight 12 kg. Entered the hospital 9 months ago with anemia and enlarged spleen. The spleen was removed and the patient made a good recovery. Subsequently the diagnosis of kala-azar was made. On account of the rarity of this condition this patient had been kept at the hospital for observation. She had been afebrile for 5 weeks previous to the beginning of our experiments and had been gaining steadily in weight.

This child did well while on the high protein diet but on the low protein diet developed a diarrhea (probably due to the large amounts of cream and butter consumed) which made the quantitative collection of urine difficult. While, therefore, it will be noticed that a marked fall in creatine excretion occurred it was not thought advisable to continue the low protein diet long enough to get minimal creatine figures.

TABLE I.

Date.	Preformed creatinine.	Creatine.	Total nitrogen.	Protein diet.
March	*mg.*	*mg.*	*gm.*	
15–16	140	180	5.0	High.
16–17	131	146	4.5	"
17–18	152	173	4.0	"
18–19	137	113	3.2	"
19–20	142	105	2.9	Low.
20–21	143	71	1.1	"
21–22	133	42	1.4	"
22–23*	...	...	...	High.
23–24	137	137	2.8	"

* Urine not quantitatively collected.

[4] Folin, O., *J. Biol. Chem.*, 1914, xvii, 472.
[5] Folin, O., and Doisy, E. A., *J. Biol. Chem.*, 1917–18, xxviii, 349.
[6] Folin and Denis, *J. Biol. Chem.*, 1916, xxvi, 473.

Subject II (*Table II*).—A boy, 6 years old, weight 17.08 kg. This child had been an inmate of the hospital for 4 months while receiving surgical treatment for osteomyelitis. At the time at which we began our experimental work he was about to be discharged, and was detained for use in our experiment.

This child took the low protein diet well, and, as will be seen from the result presented in Table II his creatine dropped from 132 mg. on the high protein diet to 3 mg. after 8 days of low protein feeding.

TABLE II.

Date.	Preformed creatinine.	Creatine.	Total nitrogen.	Protein diet.
March	*mg.*	*mg.*	*gm.*	
15–16	200	132	7.0	High.
16–17	...	...	...	Low.
17–18	199	101	2.9	"
18–19	189	77	2.4	"
19–20	189	41	2.7	"
20–21	190	26	2.2	"
21–22	191	18	1.8	"
22–23	206	9	1.4	"
23–24	213	3	1.2	"
24–25*				
25–26	211	4	1.1	"
26–27	219	73	2.6	High.
27–28	217	88	4.32	"
28–29	210	105	5.22	"

* Urine not quantitatively collected.

Subject III (*Table III*).—A boy, 6 years and 4 months old, weight 16.25 kg. This child had been an inmate of the hospital for 4 months while being treated for osteomyelitis. As in the case of Subject II he was in a late stage of convalescence and was detained merely for use as an experimental subject. The experimental results obtained on this child are of particular interest for it will be noted in the table that in only 4 days of low protein feeding his urine became creatine-free.

Subject IV (*Table IV*).—A boy, 5 years old, weight 17.0 kg. This patient entered the hospital for the removal of a keloid following a burn. He may be considered a normal child in every respect.

His stay in the hospital was brief so that we were only enabled to make use of him for 7 days; even in this short time, however, the rapid fall in creatine excretion (from 130 to 6 mg. in the course of 3 days of low protein feeding) makes the experiment of considerable interest.

Subject V (*Table V*).—An infant, 13 months old, weight 9.6 kg. This infant entered the hospital for treatment of a mild attack of bronchitis. When convalescent he was used for the experiments recorded below. He may be considered in every respect a normal child. While the fall in creatine excretion is considerable, amounting to about 50 per cent in 3 days of low protein feeding, the low figures reached in the case of some of the older children were not obtained. That this is due to the brevity of

TABLE III.

Date.	Preformed creatinine.	Creatine.	Total nitrogen.	Protein diet.
March	*mg.*	*mg.*	*gm.*	
15–16	190	60	5.8	High.
16–17	196	60	4.2	"
17–18	210	122	3.7	"
18–19	220	102	5.1	"
19–20	218	40	2.8	Low.
20–21	223	16	2.4	"
21–22	196	3	1.8	"
22–23	210	0	2.0	"
23–24*	...	...	...	High.
24–25	230	114	3.0	"
25–26	215	121	4.1	"
26–27	219	152	5.0	"

* Urine not quantitatively collected.

TABLE IV.

Date.	Preformed creatinine.	Creatine.	Total nitrogen.	Protein diet.
March	*mg.*	*mg.*	*gm.*	
20–21	200	170	5.1	High.
21–22	190	223	4.3	"
22–23	210	130	3.0	Low.
23–24	220	20	2.1	"
24–25	204	6	1.8	"

the experiment and is not in any way connected with the age of the subject is shown by the results obtained by Talbot and Gamble[7] on a 5 months old infant who received amounts of protein varying from 9.9 to 37.4 gm. and whose creatine output varied from 3 mg. on the former diet to 117 mg. on the latter. Measurements of the hydrogen ion concentration of the urine of this infant are also reported but indicate no relation between urinary acidity and creatine output, a fact of interest in view of the recent investigations of Underhill[8] on the relation of creatinuria to acidosis.

[7] Talbot, F. B., and Gamble, J. L., *Am. J. Dis. Child.*, 1916, xii, 333.
[8] Underhill, F. P., *J. Biol. Chem.*, 1916, xxvii, 127.

TABLE V.

Date.	Preformed creatinine.	Creatine.	Total nitrogen.	Protein diet.
March	*mg.*	*mg.*	*gm.*	
27–28	100	102	3.2	High.
28–29	99	56	2.8	Low.
29–30	110	81	1.3	"
30–31	99	53	1.0	"

Powis and Raper[9] have recently published the results of a series of experiments on two children in which they found that the excretion of creatine was greatest during the forenoon, decreased during the afternoon, and was very small indeed at night. This diminution of creatine excretion during the night is attributed by these investigators to "the state of rest of the skeletal muscles associated with the temporary cessation of voluntary control which occurs during sleep." Somewhat similar results have been obtained by one of us[10] with adults and with two boys aged 10 and 12 years.

In Table VI are given the results obtained by determining the creatine separately in the day and night urines of the children used as subjects for the experimental results presented above.

TABLE VI.

Subject.	Day, 8 a.m. to 8 p.m.		Night, 8 p.m. to 8 a.m.		Total nitrogen for 24 hrs.
	Preformed creatinine.	Creatine.	Preformed creatinine.	Creatine.	
	mg.	*mg.*	*mg.*	*mg.*	*gm.*
I	73	103	58	43	4.5
I	75	42	58	0	1.4
II	100	40	117	48	4.3
II	100	9	106	0	1.4
III	84	30	92	30	4.2
III	96	3	100	0	1.8
V	55	55	45	47	3.2
V	60	51	50	30	1.3

In order to save space only the results of one high protein and one low protein day are given.

[9] Powis, F., and Raper, H. S., *Biochem. J.*, 1916, x, 363.
[10] Denis, *J. Biol. Chem.*, 1917, xxix, 447.

These results are of interest for the reason that contrary to the findings of Powis and Raper they indicate clearly the important part played by food intake on creatine excretion. While in two subjects the excretion of creatine is slightly greater during the day than at night in two (Subjects II and V) the elimination of this body during the periods of high protein feeding is practically the same during the two periods. The explanation is simple. These children were given a substantial supper at 5 p.m., took food (cocoa and bread, or milk) twice during the night, and received breakfast at 7 a.m. The infant (Subject V) received the same number of feedings during the night as in the day. During the period of low protein feeding the only food given the children at night consisted of lactose lemonade, with the result that no creatine was eliminated during this time.

Three general hypotheses regarding the origin of creatinuria have been proposed:

Mendel and Rose,[11] as a result of their experiments on starving rabbits, have suggested that creatinuria is in some way closely connected with carbohydrate metabolism.

McCollum and Steenbock,[12] by an interesting series of experiments on the pig, have obtained results which point to a connection between creatinuria and protein metabolism. Recently Underhill[8] has suggested the possible connection between creatinuria and acidosis.

The results of experimental work recently published from this laboratory[3] would seem to be in accord with the findings of McCollum and Steenbock regarding the intimate relation between creatine excretion and protein intake. The experiments reported in the present paper also lend themselves readily to the same interpretation.

SUMMARY.

Experimental results are presented on four children and one infant in which it is shown that the amount of creatine found in the urine of children is directly dependent on the intake of protein, being high when large quantities of protein (creatine-free)

[11] Mendel, L. B., and Rose, W. C., *J. Biol. Chem.*, 1911–12, x, 213.

[12] McCollum, E. V., and Steenbock, H., *J. Biol. Chem.*, 1912–13, xiii, 209.

are ingested, decreasing and in some cases disappearing entirely when the child is fed a diet of an extremely low protein content. Creatinuria in normal children is therefore due to the relatively high protein intake which is the rule with practically all children; that it may also be due to the low saturation point of immature muscle is suggested by the small creatine content of the muscles of children and by the relatively low level of protein consumption at which appreciable quantities of creatine are excreted.

ENZYME AND REACTION OF MEDIUM IN AUTOLYSIS.

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It is possible, by means of cataphoresis, to demonstrate that the proteolytic enzyme of the liver cells of the guinea pig acts only in an acid medium, that is, where pH < 7.0.

The modification of the original Bechold apparatus by Michaelis[1] was used in these experiments. The cathode consisted of copper in $CuSO_4$, in the presence of a small amount of $CuCl_2$. The anode was of silver, in NaCl (AgCl). Distilled water filled the apparatus with the exception of the lower horizontal vessel, in which was placed the suspension of liver. The preparation of this suspension was as follows: The guinea pig was killed by concussion, the liver excised, and frozen within 50 seconds on a CO_2 freezing microtome stage. The liver was then ground in a mortar and taken up in the form of a suspension in 0.9 per cent NaCl. Potentiometer measurements with a Clark electrode showed a change during this time from pH = 7.2 to 6.8. Cataphoresis was maintained by means of a direct current of 110 volts for 24 hours. The temperature varied from 20 to 24°C. It was found impracticable to use the ordinary antiseptic agents, such as toluene. It was found undesirable, likewise, to place the apparatus in the thermostat room at 37.5°C., inasmuch as bacterial action was accentuated.

At the end of the period of cataphoresis, it was determined that the vessels immediately above the horizontal compartment containing the suspension of liver gave pH = 5.0 on the cathode side and that on the anode gave pH > 7.8. The ninhydrin reaction was distinctly positive on the cathode side and entirely negative on the anode. Gasometric amino-acid determinations gave,

[1] Michaelis, L., *Biochem. Z.*, 1909, xvi, 81.

on the average, 0.16 mg. of NH_2 N, per cc. in the cathode vessel; on the anode, the average reading was not greater than the correction for error.

It is evident that it is necessary to take into consideration some factors which may complicate the obvious conclusion that no hydrolysis has taken place in the anode portion.

In the first place, amino-acids are usually considered to be amphoteric compounds. This is not true in electrochemical considerations, for while the monoamino-monocarboxylic α-amino-acids have isoelectric points where pH $\lessapprox$ 6.5, lysine and arginine, and probably histidine, have the value pH $\gtrapprox$ 9.00.[2] Accordingly, the latter class of amino-acids appears at the anode, being electro-negative. Again, the objection which might be urged that these so called amphoteric compounds, being electrolytes dissociating so that neither the ninhydrin nor the nitrous acid method would reveal their presence necessarily, on the anode side, but, owing to high concentration would be detected as molecules on the acid side, is untenable.

It is to be remembered that, if autolysis proceeded in an alkaline medium, amino-acids arising in the hydrolysis unite with cations and, on ionizing, become electro-negative; thus they appear at the anode. If the enzyme, then, acted in an alkaline medium, we should expect to find a positive ninhydrin reaction given by the anodic liquid; this, however, is not the case.

It may be worth recording that the appearance of the horizontal vessel was such that it is difficult to believe in an alkaline hydrolysis. Invariably, there was a diaphragm formed somewhat to the alkaline side of the middle of the transverse vessel, which, on analysis was found to consist of hemoglobin-impregnated protein masses, together with fat. Whether this represented the isoelectric point for these compounds could not be determined, but nevertheless there was a striking difference in appearance between the tissue to the anodic side and that on the cathodic side of this diaphragm, the former showing impaction, with little or no evidence of liquefaction, while the latter showed decided liquefaction.[3]

[2] Scudder, Constants of Organic Compounds, New York, 1914.

[3] By placing 2 cc. of liquid from each of the vessels in contact with Witte's peptone solution, it was determined that the enzyme was present in both instances, but much in excess in the cathode vessel.

The relation between enzyme and reaction to medium in the case of autolysis enzymes has not been studied to any extent. The often quoted paper of Wiener[4] was written at a time when exact methods for determining reaction of medium were not available. Jobling and Petersen[5] and Jobling and Strouse[6] have reported autolysis in alkaline media, while Hedin[7] has described for the spleen, two proteolytic enzymes, one active in an alkaline medium and the other in acid. Bradley and Taylor[8] believe that according to their results, it "is proven that the enzyme is active in the neutral or amphoteric liver material." In an earlier paper, Bradley and Taylor[9] studied the problem using various reagents in different concentrations and observed the results upon rate and point of equilibrium of autolysis in mammalian liver. The writer,[10] using the Sörensen colorimetric method, attempted to determine the early relation between enzyme and medium, and is now extending the study to those cases where alkaline hydrolysis by autolytic enzymes has been definitely described.

CONCLUSION.

In the guinea pig liver, the proteolytic enzyme of autolysis is active only in an alkaline medium.

[4] Wiener, H., *Zentr. Physiol.*, 1905–06, xix, 349.
[5] Jobling, J. W., and Petersen, W., *J. Exp. Med.*, 1914, xix, 383.
[6] Jobling, J. W., and Strouse, S., *J. Exp. Med.*, 1913, xviii, 591.
[7] Hedin, S. G., *J. Physiol.*, 1903, xxx, 155.
[8] Bradley, H. C., and Taylor, J., *J. Biol. Chem.*, 1916, xxv, 374.
[9] Bradley and Taylor, *J. Biol. Chem.*, 1916, xxv, 261.
[10] Morse, M., *J. Biol. Chem.*, 1916, xxiv, 163.

THE BIOLOGICAL EFFICIENCY OF POTATO NITROGEN.

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(Received for publication, April 25, 1917.)

The most significant contribution to the literature relating to the nutritive value of potato nitrogen is that of Hindhede.[1] His experiments were planned primarily to study the minimum nitrogen requirement and the choice of the potato was due to its low nitrogen content. The work covered 309 days, divided into three periods devoted respectively to light, moderate, and severe muscular work. Three healthy adult men served as subjects. For short periods at different times the diet included, besides potatoes and "margarine," small amounts of one or more other foods low in nitrogen, such as onions, prunes, and strawberries, but most of the time potatoes and margarine were used exclusively, and as the margarine contained only 0.009 per cent of nitrogen, this food contributed very little to the total nitrogen of the diet. The results of the experiments in which only potatoes and margarine were used may be summarized as follows:

Subject.	No. of days.	Calories.	Food nitrogen.	Urinary nitrogen.	Fecal nitrogen.	Nitrogen balance.
			gm.	*gm.*	*gm.*	*gm.*
Madsen	54	3,592	6.66	7.35	1.04	−1.73
	97	4,900	8.46	7.58	1.23	−0.36
	40	4,388	6.52	5.49	1.71	−0.68
Munk	27	3,454	7.34	5.54	1.28	+0.52
Hindhede	27	2,570	2.97	2.65	0.82	−0.50

Somewhat earlier, Thomas[2] attempted to compare the biological value of a number of protein foods, among them the potato, to which he assigned a grade of 83 in a series in which milk ranked 99.7 and maize 40. The evidence does not justify one in taking the rating as in any sense final, partly because of failure to allow sufficient time to attain nitrogen

[1] Hindhede, M., *Skand. Arch. Physiol.*, 1913, xxx, 97.

[2] Thomas, K., *Arch. Physiol.*, 1909, 219.

equilibrium in a number of cases and partly because of the arbitrary nature of the formulas used to calculate the grades, but the protocols show that between 5 and 6 gm. of potato nitrogen sufficed to keep a 70 kilo man in equilibrium.

This work suggested to us the desirability of investigating more fully the nutritive value of potato nitrogen, and it seemed worth while, as a preliminary measure, to repeat these experiments under carefully controlled conditions and determine how quickly nitrogen equilibrium could be established on a potato diet with a nitrogen intake under 5 gm. per day. The subject was a healthy young woman weighing 50 kilos and doing the laboratory work connected with this problem in addition to routine school duties. The experiment extended over 10 days, during which the diet was as follows:

Period.	No. of days.	Daily food intake.				
		Potato.	Butter.	Sugar.	Total calories.	Agar-agar.
		gm.	*gm.*	*gm.*		*gm.*
I	5	1,365	56	18	1,870	10
II	3	1,500	57	41	2,038	10
III	2	1,500	57	61	2,120	10

The butter was heated, allowed to sediment, and decanted, strained through cheese-cloth, allowed to settle again, and decanted into glass jars. Analysis showed 0.0086 per cent of nitrogen. The potatoes were sampled daily, and an aliquot of the combined sample was taken for analysis, which showed 0.3309 per cent of nitrogen. The agar-agar was soaked and thoroughly washed, then taken in two portions daily, dissolved in hot water to which was added the sugar and a few drops of lemon juice. Urine and feces were collected daily, the urine was analyzed in periods of 24 hours, the feces were dried and analyzed in three periods. The results are tabulated below.

Day.	Food nitrogen.	Urinary nitrogen.	Fecal nitrogen.	Nitrogen balance.
	gm.	*gm.*	*gm.*	*gm.*
1	4.52	4.39	0.95	−0.81
2	4.52	5.27	0.95	−1.69
3	4.52	5.25	0.95	−1.67
4	4.52	3.11	0.95	+0.47
5	4.52	3.52	0.95	+0.05
6	4.97	3.93	1.22	−0.18
7	4.97	3.26	1.22	+0.51
8	4.97	3.01	1.22	+0.74
9	4.97	3.13	2.05	−0.21
10	4.97	2.97	2.05	−0.05

It is evident that nitrogen equilibrium was established by the 4th day and maintained throughout the remainder of the experiment with a total daily intake averaging 4.8 gm. (0.096 gm. per kilo), all of which was supplied by potato except 0.0047 gm. (0.1 per cent). The total available nitrogen was 3.4 gm. per day, or 0.068 gm. per kilo. The high fecal nitrogen of the last period seemed due to the shortness of the period and the difficulties of perfect separation at the close of the experiment. There were no digestive disturbances apparent. The subject did not tire of the potato diet, and said she never felt better than at the end of this experiment.

In Thomas's experiments, nitrogen equilibrium was nearly maintained with 0.08 to 0.09 gm. of nitrogen per kilo, and in his best experiment fully maintained on 0.057 gm. per kilo; in Hindhede's work, with 0.038 to 0.08 gm. (average 0.056) per kilo. It is evident that the potato will support the maintenance metabolism of the adult when from 0.05 to 0.075 gm. of nitrogen per kilo of body weight is provided.

SUMMARY.

On a diet adequate in total fuel, consisting of potatoes and clarified butter, in which the potatoes supplied all but 0.1 per cent of the total nitrogen, nitrogen balance was maintained for 7 days on a total nitrogen intake of 0.096 gm. per kilo, equivalent to a net intake of 0.068 gm. per kilo. This is in harmony with other experiments in which nitrogen equilibrium has been

maintained on potato nitrogen when the net available supply was from 0.04 to 0.08 gm. per kilo, and demonstrates that the potato is a source of nitrogen compounds of high nutritive efficiency in spite of the fact that only 63 per cent of the potato nitrogen is reported to be in the form of protein. Further investigations on the value of potato nitrogen are in progress.

THE ALLEGED NINHYDRIN REACTION WITH GLYCEROL, ETC.

By VICTOR JOHN HARDING.

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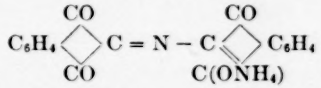
(Received for publication, April 20, 1917.)

Harding and Warneford,[1] and Harding and MacLean,[2] have examined the reaction of ninhydrin (triketohydrindene hydrate) with amino-acids, ammonium salts, amines, and amides. They found that the ninhydrin reaction was given by amino-acids even in very dilute solutions, and by all ammonium salts, provided the concentrations were sufficiently high. The reaction with amines was found to be dependent on the concentration of the amine and on its constitution, all bases of the type $R.CH_2.NH_2$, and bases of the type $R.CH(NH_2)R$ where one radicle was negative in character, giving positive results. Amides gave negative results. They attempted to build up a theory of the mechanism of the reaction which would explain why compounds so diverse as ammonium salts, amino-acids, and amines gave the same coloring matter with ninhydrin. This coloring matter was proved by Ruhemann[3] to be in the case of amino-acids the *ammonium* salt of *diketohydrindylidene-diketohydrindamine.*



The similarity of the coloring matter obtained by the interaction of ammonium salts and the classes of bases just mentioned, with ninhydrin, was established by means of its behavior in dilute solution. It showed (1) The same absorption band in the visible spectrum; (2) the same change from blue to reddish

[1] Harding, V. J., and Warneford, F. H. S., *J. Biol. Chem.*, 1916, xxv, 319.

[2] Harding, V. J., and MacLean, R. M., *J. Biol. Chem.*, 1916, xxv, 337.

[3] Ruhemann, S., *J. Chem. Soc.*, 1911, xcix, 1486.

purple when viewed in daylight and artificial light; and (3) the same resistance to oxidation by a current of air.

The theories expressed in the two previous papers showed the possibility of the formation of this coloring matter or various salts of it from the three classes of nitrogenous compounds.

Halle, Loewenstein, and Pribram,[4] have stated that glycerol gives a ninhydrin reaction. As glycerol does not contain nitrogen it is evident that this statement, if true, opens up quite another reaction, which demands investigation. Other aldehydes, ketones, and alcohols, it is claimed, show a similar behavior. All these, however, give the reaction only when in high concentration.

Our first attempts to produce the "glycerol color" resulted in failure, due, as was found later, to the glycerol containing too high a percentage of water. In subsequent attempts we were able to observe the formation of a bluish color, if the directions of Halle, Loewenstein, and Pribram were carefully followed. It was soon found, however, that different specimens of glycerol gave very varying amounts of color. A specimen of Schuchardt's "crystallizable" glycerol gave only the faintest pink tinge when heated with ninhydrin. Another marked "anhydrous" gave quite a strong blue color. The color in this latter case was strong enough to give the three tests mentioned in the previous paper, and so was identified as a salt of *diketohydrindylidene-diketohydrindamine*. This compound, of course, could not arise from glycerol. Its source must have been some nitrogenous impurity.

That the amount of nitrogenous impurity necessary to produce a deep blue color under these conditions is very small is evident from the following experiment. A test-tube was thoroughly rinsed with ammonia-free water and allowed to drain, 1 cc. of ammonium chloride solution containing 0.05 mg. of nitrogen was next pipetted into the test-tube and then allowed to drain away. 0.2 cc. of 1 per cent ninhydrin solution was then placed in the tube, 2.0 cc. of Schuchardt's crystallizable glycerol, which previously had only given the faintest reaction by itself, were added, and the mixture was boiled over a free flame for 30 seconds, thus duplicating the conditions given by Halle, Loewenstein, and Pribram. A strong blue color developed, which was identi-

[4] Halle, W., Loewenstein, E., and Pribram, E., *Biochem. Z.*, 1913, lv, 357.

fied by the usual tests as due to a solution of the *ammonium* salt of *diketohydrindylidene-diketohydrindamine*.

The amount of ammonium chloride must under these conditions have been exceedingly small, and yet the coloration produced was much stronger than that given by any glycerol specimen which we have examined. Also it is to be noted that ammonium chloride under ordinary conditions gives no coloration with ninhydrin so that the coloration under these conditions is to be attributed to the high temperature and to the reducing action of the glycerol. This reducing action of the glycerol and the consequent increase in the formation of the coloring matter was first pointed out by Harding and Warneford. These authors also pointed out the reducing action of glycol, glucose, and *p*-oxybenzaldehyde upon ninhydrin, and the subsequent increase in the ninhydrin reaction with ammonium salts. In consequence of these facts it cannot be concluded that substances of these types give of themselves a positive ninhydrin reaction. It is much more likely that the ninhydrin reaction can be used to detect nitrogenous impurities in these classes of substances, even when present in amounts too small to be detected by ordinary reagents.

It is to be noted that the "glycerol color" as claimed by Halle, Loewenstein, and Pribram is intensified by alkali, and that it is not produced in the cold on standing in a desiccator, or on heating in a vacuum of 3 mm., the two latter conditions, in their opinion, showing the necessity of oxygen in the formation of the color. In contradistinction, the ninhydrin reaction with amino-acids is unaffected by alkali, and being produced in absence of air is held to be formed without the aid of oxygen.

A critical examination of the experimental conditions used by these authors, in the light of the investigations in these laboratories, shows that such conclusions are by no means permissible. In fact the results are decidedly in harmony with the ideas expressed in our previous papers on this subject. In all the experiments of Halle, Loewenstein, and Pribram the glycerol or the amino-acid was in excess of the ninhydrin. This would inevitably lead to the reduction of the greater part of the triketone to diketohydrindol or to hydrindantin. In the case of glycerol the small amount of reactive nitrogenous impurity would pro-

duce a small amount of diketoketohydrindylidene-diketohydrindamine (as a salt) leaving the excess of hydrindantin or diketohydrindene to give a blue chromo salt with alkali. The nitrogenous impurity (most likely an ammonium salt, having regard to the method of manufacture and purification of glycerol) would only react at a high temperature when assisted by the reducing action of the glycerol, as its amount must be far below the limits of the reactivity of ammonium salts or amines studied by Harding and Warneford and Harding and MacLean.

By heating the glycerol in a vacuum of 3 mm. it is highly probable that the boiling point of the glycerol would be so lowered as to be below the decomposition point of the nitrogenous impurity, and so the reaction would fail to show in so delicate a form.

On the other hand, the reaction with amino-acids could take place under all these conditions; the dissociation into ammonia and glyoxal being supposed to take place at ordinary temperatures, and the amino-acid being in excess of the ninhydrin, a blue color would be produced, unaffected by alkali. Thus it is difficult to see in the ninhydrin reaction with glycerol any new class of reaction.

SUMMARY

It is believed that the glycerol color given by ninhydrin and glycerol, as claimed by Halle, Loewenstein, and Pribram, is due to the presence of nitrogenous impurities in the glycerol.

Since writing the foregoing a paper has appeared criticizing the views of the author on the mechanism of the ninhydrin reaction in general.[5] It may be pointed out, however, that the coloring matter produced in the ninhydrin reaction with amino-acids was proved to be a salt of diketohydrindylidene-diketohydrindamine by Ruhemann by actual isolation and analysis of the compound. Retinger's view that the coloring matter consists of salts of hydrindantin cannot be admitted in its entirety as salts of this substance show no absorption in the visible spectrum.

[5] Retinger, J. M., *J. Am. Chem. Soc.*, 1917, xxxix, 1059.

IMPROVED CHEMICAL METHODS FOR DIFFERENTIATING BACTERIA OF THE COLI-AEROGENES FAMILY.*

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INTRODUCTION.

When determined accurately by a method such as that of Keyes (1909) the ratio of the gases CO_2 and H_2 liberated in the anaerobic fermentation of dextrose by bacteria of the coli-aerogenes family has proved to be an excellent basis for the differentiation of these organisms. The ratio for about 660 cultures has now been carefully determined in this laboratory. In their study of cultures isolated from milk and milk products, Rogers, Clark, and Davis (1914) found that approximately half gave a low gas ratio. On media more appropriate than that used in this first investigation the ratio is $\frac{CO_2}{H_2} = 1.06$, which is identical with that found by Keyes and Gillespie (1912) in their study of *B. coli* in "synthetic" media. The other organisms from milk gave decidedly higher and more variable ratios. Taking up a search for possible sources of these two groups which were found in milk, Rogers, Clark, and Evans (1914) found that of 150 cultures isolated from bovine feces all but one were of the low ratio type. These authors (1915) then reported that of 166 cultures isolated from grain 150 were of the high ratio type. Recently (Rogers, Clark, and Lubs, 1916) it has been found that special methods can reveal the presence of the high ratio type in human feces and presumably in bovine feces, but that the predominating organisms of the coli-aerogenes family which are

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found in human feces as well as in bovine feces are of the low ratio type.

The significance of these observations made it imperative that some test should be found which would correlate so well with the gas ratios that it could be used in routine investigations in place of the laborious and time-consuming gas analyses. Such a test was worked out by Clark and Lubs and published in a previous paper (1915). We have now to report some improvements in this and the Voges-Proskauer tests.

For the sake of brevity and convenience and without committing ourselves to the classification, we shall call those organisms which furnish the low gas ratio *B. coli* and those which furnish a high gas ratio *B. aerogenes*. In the latter group there are doubtless included several species such as *B. cloacæ*.

The Principle of the Methyl Red Test.

This test is based upon the difference in the acid production of the two groups of the colon-aerogenes family.

A coli culture if furnished sufficient dextrose will continue to elaborate acid until a limiting zone of hydrogen ion concentration is reached (Michaelis and Marcora, 1912; Clark, 1915). Into this zone certain cultures of the aerogenes type may penetrate if all the conditions are properly set, but to do so an aerogenes culture must be provided with much more dextrose than is required by a coli culture. An aerogenes culture may easily be prevented from reaching the relatively high hydrogen ion concentration attained by *B. coli* if its dextrose ration is limited and properly adjusted to the acid-neutralizing power of the medium or to what is technically known as the "buffer action." When such an adjustment is properly made, coli cultures will find sufficient sugar to attain a high and more or less characteristic hydrogen ion concentration near pH = 5.0, while aerogenes cultures will be forced to leave the medium less acid.

The difference in the hydrogen ion concentrations may then be easily detected with a proper indicator. Since in the special medium adopted coli cultures appear red and aerogenes cultures yellow on the addition of methyl red, the test has come to be known as "the methyl red test." Any other indicator with a pH range similar to that of methyl red may be used.

The differences in hydrogen ion concentration may be intensified by taking advantage of another phenomenon. When either a coli or an aerogenes organism has brought its medium to a hydrogen ion concentration in the neighborhood of pH = 5.0, there is a tendency for the reaction to stick there. If, on the other hand, this lethal zone has not been reached, the medium may undergo a reversion of reaction toward alkalinity. Now with a medium properly adjusted in the relation of dextrose content to buffer action, a coli culture can be brought to the lethal zone while a high ratio culture is left at a hydrogen ion concentration favorable to the reversion of reaction. Consequently as time goes on the hydrogen ion concentrations in the two cases become further apart.

A medium which was found suitable for this differential test is composed of 0.5 per cent dextrose, 0.5 per cent K_2HPO_4, and 0.5 per cent Witte peptone (Clark and Lubs, 1915).

The Revision of the Medium.

Because of the present scarcity of Witte peptone numerous investigators have inquired regarding the substitution of other peptone preparations in the medium used in the differential test.

We have made but one set of experiments in which an American peptone preparation was substituted for Witte's. In these experiments we noted a few anomalies which were immediately traced to the unique buffer action of the "peptone" used. Titration curves of some other "peptones" were then made and it was seen that the indiscriminate substitution of these would inevitably lead to uncertain results. There is no apparent reason why any one of the peptones which we have studied cannot be used in the medium for the methyl red test provided the composition of the medium is properly readjusted; but rather than cover this ground again with only new peptone preparations as variations we determined to construct a suitable medium from well defined crystalline compounds.

So far as the provision of a utilizable source of nitrogen is concerned, there is no difficulty in replacing Witte peptone, since the organisms with which we are now concerned are not fastidious. The Witte peptone, however, takes part in the buffer

action, the adjustment of which to the dextrose content is absolutely essential to the success of the test. Were the medium left with the buffer action of the phosphate alone there would remain a weak buffer action between pH = 6 and pH = 5. This zone is the critical one and if left unbuffered will lead to uncertainties in the test. It is, therefore, necessary that the Witte peptone be replaced not only by a proper source of nitrogen but by a proper buffer.

Since phthalate buffer mixtures (Clark and Lubs, 1916) overlap the pH zone covered by phosphate mixtures and are active in the zone pH = 6 to pH = 5, it was decided to provide the desired buffer action by adding to the medium acid potassium phthalate.

Instead of the K_2HPO_4 used in the original medium the sodium salt was chosen because it may be obtained in crystalline form.

Aspartic acid was selected as the source of nitrogen because it is less easily decomposed during sterilization than the more frequently used asparagine. It is easily recrystallized and readily utilized, and it forms colored complexes with dextrose less readily than ammonium salts.

These three substances, aspartic acid, di-sodium hydrogen phosphate, and acid potassium phthalate must be adjusted in their relative concentrations so that a proper initial hydrogen ion concentration may be obtained. It was decided to give the medium an initial neutral reaction not because this is necessary for good growth but because it is considered appropriate to sterilize the medium at a nearly neutral reaction. At more alkaline reactions there are formed colored complexes between the sugar and the nitrogenous body. Inspection of the curve for phosphate buffer mixtures shows that a mixture of seven molecular parts of the basic phosphate with three molecular parts of the acid constituents will give a pH value not very far from the neutral point. A rough calculation indicated that if the medium were given the percentage composition 0.7 per cent Na_2HPO_4 (anhydrous), 0.2 per cent KH phthalate, 0.1 per cent aspartic acid, it would be approximately neutral and such it was found to be. A 0.4 per cent dextrose content was determined upon after some preliminary empirical tests with organisms of both groups.

The "titration curve" of this medium is shown in Fig. 1. In this figure the quantities of 0.1 N lactic acid added to 100 cc. portions of the medium are plotted as abscissæ and the corresponding pH values as ordinates. In the same figure is shown the titration curve of the old medium. It will be noted that the new medium is more heavily buffered in the region pH = 5 to pH = 6 than is the old medium. At first this seemed unnecessary and we have sought to modify it and at the same time keep the proper proportions of acid and basic constituents so that

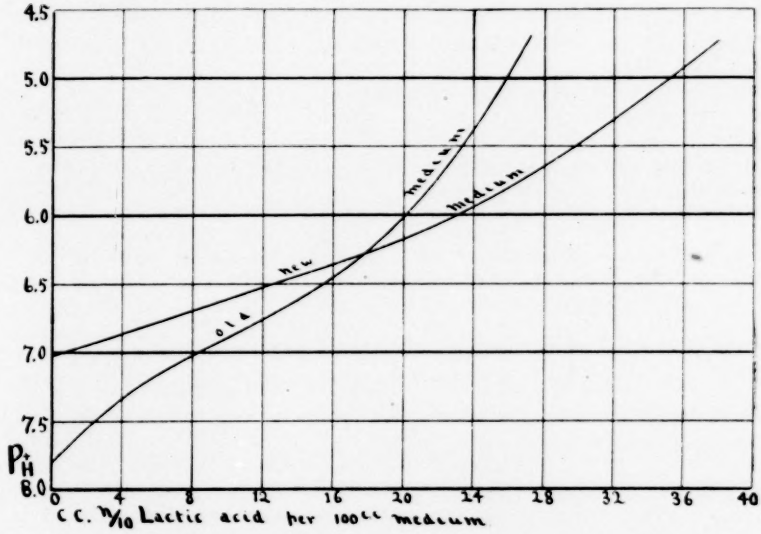

FIG. 1. Titration curves of old and new media for the methyl red test.

the initial neutral reaction may be retained. These experiments have revealed some indications that the aspartic acid content cannot be varied at will without injecting some complicating phenomena connected with the attack upon this substance and we have, therefore, fallen back upon the medium as it was first designed.

Since this medium is the one we have used in an extensive series of tests and which we recommend for trial by others, the preparation of its constituents will be described.

Preparation of Material.

Di-Sodium Hydrogen Phosphate (Na_2HPO_4).—This crystallizes from its aqueous solutions at room temperaure with twelve molecules of water of crystallization. This water of crystallization weighs more than the anhydrous salt itself, a fact which may be emphasized because in describing the use of this salt bacteriologists frequently have failed to specify the form in which it is to be used. The crystals containing twelve molecules of water of crystallization effloresce easily, although according to Faraday (1839) similar crystals may be kept intact indefinitely if not injured. Ordinarily a more stable form is desirable for weighing. In our own work, we used the anhydrous salt obtained by heating the crystals for a day at 110° *in vacuo* over H_2SO_4 (Schmiedeberg desiccator). Since the anhydrous salt absorbs moisture with great avidity it is preserved in small sealed bottles. Sörensen (1909) has described a stable form of the salt containing two molecules of water of crystallization. To prepare this take some of the pure material which has been recrystallized two or three times, grind it, and spread it in thin layers between filter paper. Expose this to a clean atmosphere for about 2 weeks, when the salt will have lost all but two molecules of water of crystallization.

The molecular weights of the three forms are:

$Na_2HPO_4.12H_2O$	358.24
$Na_2HPO_4.2H_2O$	178.08
Na_2HPO_4 (anhydrous)	142.05

To obtain a 0.7 per cent solution of Na_2HPO_4 (anhydrous) use 0.88 per cent $Na_2HPO_4.2H_2O$.

Acid Potassium Phthalate.—This may be prepared by the method of Dodge (1915) modified as follows. Make a potassium hydroxide solution by dissolving about 60 gm. of a high grade sample in about 400 cc. of water. To this add 50 gm. of the resublimed anhydride of ortho-phthalic acid. If a diluted portion of this solution is still alkaline to phenolphthalein add more phthalic anhydride; if acid add more alkali. When the solution is thus roughly adjusted add as much more phthalic anhydride as the solution contains and heat till all is dissolved. Filter while hot and allow the crystallization to take place slowly. The crystals should be drained with suction and recrystallized at least twice from distilled water. Dry at 110–115°C. to constant weight. The salt is an invaluable substance for

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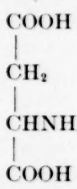
[1] The phthalic anhydride which we have recently found on the market is very impure. At least five recrystallizations of the phthalate were necessary to insure a usable product.

In ordinary times the materials used in our medium may be obtained cheaply and in high purity but for the present and until our chemical industries become stable it is essential that bacteriologists examine most carefully every chemical used in their studies.

the standardization of alkali solutions (Dodge, 1915) and it is therefore profitable to prepare quantities of it with great care. Phthalic acid may be recovered from the mother liquors by acidifying them with HCl.

Aspartic Acid.



The levo form is the isomer generally encountered. It may be recrystallized from distilled water. Its preparation from *l*-asparagine may be carried out according to the method of Schiff (1884). To 500 cc. of distilled water add one mol. of asparagine (150 gm.) and two mols. of HCl (166 cc. of 37 per cent hydrochloric acid solution). Heat the mixture in a flask with a reflux condenser over a hot plate or gentle flame for 3 hours. Then add one mol. of NH_3 (68 cc. of ammonia water of specific gravity 0.90). Filter the crystals with suction, wash with ice cold water, and recrystallize from distilled water. There is doubtless formed during the heating of the *l*-asparagine with HCl some *d*-aspartic acid, but material prepared in this manner is suitable for the medium under discussion.

Dextrose.—In our own work we have used a sample of dextrose kindly prepared for us by Dr. Hudson of the Bureau of Chemistry. This sample had been recrystallized from acetic acid. The method is given in detail in a recent paper by Hudson and Dale (1917).

Methyl Red Solution.—Dissolve 0.1 gm. of methyl red (ortho-carboxybenzene-azo-dimethyl-aniline) in 300 cc. of redistilled alcohol and dilute to 500 cc. with distilled water.

Certain samples of methyl red which have come within our knowledge have been grossly impure. If such a sample be encountered it should either be discarded or recrystallized from benzene.

Materials less carefully purified than those described are doubtless permissible. Indeed the medium could not be recommended unless a reasonable degree of tolerance in the purity of the materials were permissible; but it is hardly worth while describing a medium of this nature unless it is well defined and is reproduced by others with a reasonable degree of exactness. At the present state of affairs revealed in recent discussions by various local sections of the American Chemical Society no reliance is to be placed upon the analyses of chemicals stated on the labels by several manufacturers. Consequently no reliance can be placed upon any cultural tests unless there is given some indication of the purity of the chemicals used.

It is of course well recognized that a substance prepared by a few recrystallizations of commercial material with the use of ordinary glassware and under ordinary conditions is not strictly speaking perfectly pure.

The materials which we have used in the preparation of our medium may therefore have contained elements other than C, H, O, N, P, Na, and K. However, it is hardly to be expected that other elements or other compounds were present in sufficient concentration to play a decisive rôle in the vigorous growths observed. It is true that in most cases inoculations were made from agar slopes containing, beside the impurities of the agar, some Liebig's extract of beef. There was thus transferred an appreciable quantity of the material of the agar medium. But we have made daily transfers from one tube of the synthetic medium to another by means of a clean platinum needle and observed no diminution in the growth or vigor of fermentation by the four strains so studied.

Procedure.

For 1 liter of the medium dissolve in about 800 cc. of hot distilled water 7 gm. of Na_2HPO_4 (anhydrous) or 8.8 gm. of $Na_2HPO_4 .2H_2O$, 2 gm. of KH phthalate, 1 gm. of aspartic acid, and 4 gm. of dextrose. When the materials are completely dissolved cool the solution to 20°C. and make it up to 1,000 cc. with distilled water.

Apportion the medium in approximately 10 cc. portions in dry-sterilized glass test-tubes and sterilize in an autoclave for 15 minutes after the steam pressure has reached 15 pounds. Inoculate from pure cultures and incubate at 30°C. for 3 to 5 days.

Levine (1916, *a*) has suggested an incubation temperature of 37°. This we have avoided in our own work because several of the cultures in our collection will not grow well at the higher temperature.

Most of the tests we have made were with cultures incubated for 5 days. Experiments with about half the cultures indicated that a 3 day period is sufficient, but the reliability of this shorter period remains to be determined by more extensive experiments.

For making the test with methyl red in a gross way, it is only necessary to add five drops of a 0.02 per cent solution of the indicator. With methyl red the coli cultures will appear red and the aerogenes cultures yellow. It is preferable for careful work to measure the pH values with methyl red and bromocresol purple, using standard solutions and the procedure we have described elsewhere (Clark and Lubs, 1917). It is also well to save a portion of each culture for the Voges-Proskauer reaction.

The Voges-Proskauer Reaction.

By the work of Harden (1899, 1901, 1905, 1906), Harden and Walpole (1906), and Thompson (1911) there was established in a limited number of cases a correlation between the Voges-Proskauer reaction and the gas ratios. Recently Levine (1916) in studying his extensive collection of coli-aerogenes cultures discovered an almost perfect correlation between the differentiations made with the Voges-Proskauer reaction and those made with our indicator test. By means of this correlation Levine was able to show that some of the conclusions regarding the distribution of bacteria of the colon and aerogenes types which had been reached in older studies with Voges and Proskauer's reaction were confirmed by the more careful work of Rogers and his collaborators.

It may be of interest to note that Rogers, Clark, and Davis (1914) planned to include the Voges-Proskauer test in their study but rejected it with the statement that "the reaction should be studied until the test can be made accurately and under exact conditions." It is now found that the notes of this earlier work contain records of a few tests and that there is a very good agreement between these tests and the gas ratios of the organisms studied. This correlation was overlooked before the publication of Levine's work, because in our hands the test for the Voges-Proskauer reaction was very unsatisfactory and was rejected pending some investigations started by Dr. Walters but left unfinished by his transfer to another bureau of this department.

It has now occurred to us that the Voges-Proskauer reaction may be made more satisfactory if the test is carried out in the colorless synthetic medium described in this article.

According to Harden (1906):

> "Voges and Proskauer's reaction . . . appears to be due to acetylmethylcarbinol, which is formed by the action of the bacteria on the glucose of the medium. In the presence of potash and air this is oxidized to diacetyl, which then reacts with some constituent of the peptone water."

Since the revised medium we have described contains no peptone or any protein body except such as may be formed by the synthetic action of the bacteria, it is necessary to supply some such substance at the time the test is made. Harden and Norris (1911) have shown that various proteins and peptones react with diacetyl in the presence of alkali to give the eosin-like fluorescent color characteristic of the Voges-Proskauer reaction.

Since casein can easily be obtained in a high state of purity and reacts with diacetyl it is a suitable reagent for the present purpose. We have accordingly made the test in the following manner.

To 5 cc. of a culture is added about 1 cc. of a *fresh* 0.2 per cent solution of sodium caseinate made by dissolving casein (Hammarsten preparation) in dilute NaOH. This is followed by 10 cc. of 10 per cent NaOH solution. Generally within an hour or two a pink layer develops near the meniscus and gradually spreads downward. If the fading of the color does not overtake its development, the tube within 24 hours will become suffused with a brilliant fluorescent color. The color may be intensified in some cases and destroyed in others by the use of oxidizing reagents.

In our past experience the Voges-Proskauer test has been very unsatisfactory; but when conducted in the manner just described it is generally very brilliant. There are, however, cases in which the test leaves much to be desired.

There is some evidence that the production of acetyl methyl carbinol is the result of secondary reactions, perhaps synthetic to some extent (Harden and Norris, 1912). If so its production may not be intimately connected with the main course of the fermentation and the quantity produced in any given case may not be comparable with the quantity of the main products. Harden and Norris (1911) found that the yield of the associated glycol was decidedly less in their cultures than in cultures previously studied by Harden and Walpole (1906). With the comparatively crude colorimetric test we have found but very slight indications of a positive Voges-Proskauer reaction in certain aerogenes cultures in which an otherwise vigorous fermentation had taken place.

The Organisms Tested.

There were available for the tests 245 cultures whose gas production had been carefully studied by the methods described by Rogers, Clark, and Davis, and Rogers, Clark, and Evans. Table I will show the sources and distribution by gas ratios of the organisms studied.

By "typical low gas ratio cultures" we mean those which when grown *in vacuo* upon 10 cc. of a medium consisting of 1 per cent Witte peptone, 1 per cent dextrose, and 0.5 per cent K_2HPO_4

have given a gas ratio approximately $\frac{CO_2}{H_2} = 1.06$. In some instances the ratio has deviated from this average value but never has approached the high values given by what we call collectively the aerogenes cultures. The gas volumes have been generally about 9 to 15 cc., but are subject to greater variation than the gas ratio.

In the group of typical high ratio cultures are included all those organisms which give a gas ratio higher than 1.70 and a gas volume, under the conditions mentioned, higher than 20 cc.

Thus divided, the "typical" cultures fall into two very distinct groups. The "atypical" cultures will be treated in a section by themselves.

TABLE I.

Source.	Total No. of cultures.	Typical low gas ratio cultures.	Typical high gas ratio cultures.	Atypical cultures.
Milk and cheese	4	2	2	0
Bovine feces	16	15	1	0
Human feces	175	130	45	0
Grains	15	3	12	0
Water	31	14	4	13
American Museum	4	0	1	3
Totals	245	164	65	16

The Results with "Typical" Cultures.

The 164 cultures which were thus classified definitely as low ratio cultures by the gas analysis were all positive to the methyl red test with the new medium. All gave a negative Voges-Proskauer test.

The 65 cultures which were classified as typical high ratio cultures by the gas analysis were all negative to methyl red with the new medium. All gave a positive Voges-Proskauer test.

If we include those cultures which were definitely classified by gas analysis and tested in the old medium but not in the new, we now have a total of 374 cases in which there has been a perfect correlation between the gas ratios and the methyl red tests.

The correlations between gas ratios, methyl red tests, and

Voges-Proskauer reactions among the cultures definitely classified by gas analysis we have just recorded as perfect, but it must be admitted that, if the Voges-Proskauer tests had not been made in a way which excluded the expectancy of a positive test in any instance, the recording of a positive reaction would in a few instances have been perilously close to an act of imagination. We were careful to begin observations after 1 hour and then at various periods until 24 hours had elapsed. In this way we sometimes noted a very faint positive reaction which had not appeared at first and which later faded out. Very likely we have not been using the most favorable procedure, but the very intense color which develops in most cases leads us to believe that in other instances the cultures produce very small quantities of the substance responsible for the color. If so, this qualitative test, like many another qualitative test, must be considered as more or less unsatisfactory until its quantitative aspects have become more thoroughly known or until the conditions for the formation of acetyl methyl carbinol have been more exactly defined.

pH Values Observed.

In an extensive series of tests which have been made with the medium described in our first paper it was found that the pH values obtained with coli cultures lie almost universally in the region pH = 4.8 − 5.2. With the new medium it is found that these same cultures seldom reach an acidity as high as pH = 5.0 and that the values lie between pH = 5.0 and 5.3.

We meet here a phenomenon noted by one of us (Clark, 1915) in an earlier paper. In extending the work of Michaelis and Marcora (1912) to cover a greater variety of coli cultures and a greater variety of media it was found that coli cultures did not attain any one final hydrogen ion concentration. Instead the final hydrogen ion concentrations were found to lie in a comparatively narrow zone, although in any one medium the final hydrogen ion concentrations are generally grouped about one sharp point. Whether the cultures in different media attained a higher or lower point in this zone was found to be, to a greater or lesser extent, a function of the total buffer action of the media. The greater the total buffer action, and consequently the more exten-

sive and prolonged the fermentation, the higher was the final pH value.

As emphasized in a former paper (Clark, 1915) this relationship is only rough and for various reasons of which we catch glimpses as investigations proceed it cannot be expected to be rigid. Our contention from the first has been that the acid fermentation ceases within a comparatively narrow *zone* of hydrogen ion concentration and not at some particular point in all media.

If Michaelis and Marcora (1912) intended it to be understood that a sharp point is characteristic, we cannot agree. This we believe was not their intention. The essential practical aspect is that the limiting of the fermentation is a function of the hydrogen ion concentration and not a direct function of what is known as the titratable acidity. If this is so, there certainly is a "physiological constant" involved, although it would be superficial indeed to seek for this by a mere observation of the final pH values and without consideration of time and other factors.

Cole and Onslow (1916) have asserted that they find values quite at variance with those found by Michaelis and Marcora and by us but they have not given any data to reveal either the reason or the significance of their observations.

Atypical Cultures.

In our first paper on the methyl red test we recorded some experiments with atypical cultures. By atypical cultures in this instance are meant those which cannot be definitely classified by the gas analyses because their gas ratios, gas volumes, or both are far from the values typical of our low and high ratio groups. In this newer work we have encountered several such organisms, thirteen of them among water cultures isolated by Mr. Rogers (Table I).

For purposes of comparison let us first quote the average values of the gas analyses previously published.

	Total gas. cc.	Ratio. CO_2:H_2.
Average of low ratio cultures (feces series)....	14.09	1.06
" " high " " (grain "	29.50	2.36

The "atypical" organisms are tabulated in Tables II to IV.

TABLE II.

Atypical Cultures with Abnormally Low Gas Volumes and Atypical Ratios.

Source.	Designation.	Gas volume.	Gas ratio CO_2: H_2.	Indicator test pH.	Voges-Proskauer reaction.
		cc.			
American Museum.....	22og	0.9		5.2	−
Water.....	W2b	2.0	5.60	5.8	+
"	W5b	6.0	1.25	5.3	−
"	W5d	2.9	1.28	5.2	−
"	W8g	1.3	1.51	5.2	−
"	W8j	6.7	3.71	5.8	+

TABLE III.

Atypical Cultures with Typical High Gas Ratio but Abnormally Low Gas Volume.

Source.	Designation.	Gas volume.	Gas ratio CO_2: H_2.	Indicator test pH.	Voges-Proskauer reaction.
		cc.			
American Museum.....	22of	18.3	2.28	6.0	+
Water.....	W5a	14.8	2.90	5.6	−

TABLE IV.

Atypical Cultures with Intermediate Ratios and Abnormally Low Gas Volume for High Ratio.

Source.	Designation.	Gas volume.	Gas ratio CO_2: H_2.	Indicator test pH.	Voges-Proskauer reaction.
		cc.			
American Museum.....	22oh	16.7	1.47	5.8	+
Water.....	W2c	15.6	1.42	5.7	+
"	W9a	14.8	1.38	5.5	+
"	W9b	14.7	1.42	5.6	+
"	W9c	15.6	1.31	5.6	+
"	W9d	11.0	1.32	5.6	+
"	W9e	18.6	1.27	5.6	+
"	W9i	13.7	1.45	5.4	+

Inspection of Tables II to IV will show some interesting data upon which it might be permissible to speculate; but our brief study of these organisms which we have called "atypical" has led us to believe that a more intensive study of a larger collection

of similar organisms will be necessary before a discussion is of much value.

Here we may again note the fact that when the gas ratio and gas volume are normal the methyl red test, the Voges-Proskauer reaction, and the gas ratio all correlate perfectly, but that when a perfectly clear-cut correlation fails there is generally found some abnormality in gas production. Since this is so clearly the case it is obvious that careful gas analysis remains the key to the situation.

It will be noticed especially in Table IV that several of the "atypical cultures" give in the new medium for the methyl red test pH values which are intermediate between those characteristic of the typical high and typical low ratio cultures. If there sometimes occur intermediate pH values which give neither a distinct red nor a distinct yellow color with methyl red, the question arises as to how frequently they will be encountered, for instance in water analyses, and thereby produce confusion in routine work. To throw some light upon this we tested an additional 112 cultures which had been isolated from water by Mr. Rogers. In these tests we encountered only one intermediate pH value with the new medium.

Among the intermediate pH values shown in Table IV, six were obtained with organisms isolated from the same spring.

The gas production of the 112 water cultures mentioned above have not been studied, and consequently their classification by the gas ratios is unknown; but arguing from past experience, the very clean-cut differentiation which is obtained by the methyl red test in every typical case and in all but one of these cases, the very good correlation between methyl red and Voges-Proskauer differentiations in typical cases, and the almost perfect correlation in these cases, the presumption is that all but one of these 112 water cultures can be definitely classified by the methyl red test.

Whether or not "atypical" strains will frequently be encountered in other collections remains to be seen. Whether in larger collections there will appear organisms similar to those of Table IV and having such uniform and such distinct qualities that they may be classified in a new group remains to be determined by future studies in which gas analysis may profitably be considered to be fundamental.

The Reversion of Reaction.

As pointed out in our former paper either a coli or an aerogenes culture if not brought into the lethal zone of hydrogen ion concentration near pH = 5 will, with the exhaustion of the sugar, continue its action upon the medium and progressively lower its hydrogen ion concentration (increase pH). By taking advantage of this the aerogenes cultures can be made to differ very widely from the coli cultures in the final reaction.

We have specifically stated (Clark and Lubs, 1915) that this reversion should not be attributed to the production of ammonia. It is perfectly true that under certain conditions the reversion is accompanied by an increase in ammonia but the amount of this is not sufficient to account for the degree of the reversion. Kligler (1916) has evidently overlooked our statement for he has proceeded on the assumption that the reversion is due to an attack upon the peptone (of the old medium), and he has led us to believe that he would ascribe the reversion to the liberation of ammonia. It may be shown in a very simple way that this view of the situation is entirely inadequate.

If we substitute $(NH_4)\ H_2PO_4$ for the aspartic acid in the medium described in this paper the organisms will have to derive their nitrogen supply originally from an ammonium salt alone. Later they may liberate ammonia from the hydrolytic products of their own proteins. We can, however, lower the content of total nitrogen to a point where its participation in any form in changes of the reaction of the medium will become insignificant and relieve the experimental determinations of ammonia from any criticism based on possible errors of analysis. It can then be shown that an extensive reversion of reaction can take place without any significant participation of ammonia.

In the medium, which we shall describe below, the addition of 0.062 per cent $(NH_4)\ H_2PO_4$ furnishes nitrogen, after the medium is sterilized, in a concentration of approximately M/200. Let us imagine that *all* this nitrogen becomes bound during that phase of the fermentation in which the acidity is increasing and that it is bound in such a way that it cannot dissociate and affect the reaction of the medium. Then let us assume that *all* this nitrogen is liberated as the alkali NH_4OH and that in this process there is left no acidic substance with which the alkali can pair off

such as would be set free by the deaminization of an amino-acid or an acid amide. This would be practically equivalent to the addition of ammonia until its concentration reaches M/200. If this quantity of ammonia were added to a solution composed of 0.88 per cent $Na_2HPO_4.2H_2O$, 0.2 per cent KH phthalate, 0.062 per cent (NH_4) H_2PO_4, and 0.4 per cent dextrose, the composition of which is very similar to the medium whose titration curve is shown in Fig. 1, it could only lower the reaction from pH = 6.0 to about pH = 6.3, as shown by the titration curve. This calculation indicates quite closely the reversion which would be expected from the impossibly extreme assumptions made above. There may be some suspicion that the buffer effect of the fermented medium may be somewhat less than that shown by the titration curve of the unfermented medium, so we shall raise the phosphate from 0.88 per cent to 1.0 per cent to "make assurance doubly sure," and still treat our experiments as if the buffer action were that of the weaker solution.

Now what was actually found to take place was this. An aerogenes culture, aj, in 1.0 per cent $Na_2HPO_4.2H_2O$, 0.2 per cent KH phthalate, 0.062 per cent (NH_4) H_2PO_4, and 0.4 per cent dextrose brought this medium from its original reaction of pH = 7.1 to pH = 6.0 in 48 hours and bound approximately one-half the ammonia. In 18 days the reaction had reverted to pH = 6.6, which is double that which could be accounted for on the assumptions stated above. At the same time there appeared to be a nitrogen equilibrium for there was no increase in ammonia detected by the Folin method.

To attribute the reversion of reaction to the liberation of ammonia is, therefore, impossible in this case. In the aspartate medium an increase in ammonia may accompany the reversion of reaction but the amount liberated is found to be inadequate to account for the extent of the reversion.

From the work of Ayers and Rupp (1915) and such data as we have presented it appears probable that the reversion of reaction is due in large measure to the destruction of organic acids which without doubt is taking place while they are being produced. The cessation of a rapid acid production permits the slow destruction to become apparent. Similar phenomena are reported in the literature on other themes.

A reversion of reaction toward alkalinity after an initial acid production is by no means limited to coli-aerogenes cultures. Numerous instances occurring in cultures of various organisms are on record. The cause is doubtless different in diverse cases and the degree of the reversion may vary not only with the organism but with the medium; but a clear distinction should be drawn between a reversion of reaction measured by titration with an alkali to the zone of hydrogen ion concentration where some particular indicator such as phenolphthalein changes color and a reversion measured by a change in hydrogen ion concentration. For instance, it is commonly understood that *B. proteus* produces in milk a slight initial acid fermentation and then a more vigorous and extensive "alkali production." This deduction has been drawn from the color changes in litmus milk. The phenomenon can be observed better with milk colored with dibromo-ortho-cresol-sulfonephthalein which we have recommended (Clark and Lubs, 1917) as a substitute for litmus in such cases. An apparent contradiction is found in a statement by Kendall, Day, and Walker (1914). In describing the action of *B. proteus* on milk, these authors say:

> "Contrary to what might be expected in the presence of such active proteolysis, the reaction becomes progressively acid in spite of the rapid peptonization. This might be attributable to the liberation of acid phosphates following the decomposition of the casein or to a partial decomposition of the butter fats with the liberation of fatty acids."

Kendall, Day, and Walker are speaking here of the "reaction" determined by titration. An *increase* in this value is not at all incompatible with a *decrease* in hydrogen ion concentration. For example: We inoculated a sample of sterilized milk with *B. proteus* and held it at 30° until the casein was almost all digested. The solution was made up to its original volume with distilled water to replace loss by evaporation and the hydrogen ion concentration and titratable acidity determined. The hydrogen ion concentration and titratable acidity of a control sample of the same milk were also determined. Titrations were made with phenolphthalein as the indicator, and the values obtained are expressed below in cc. of 0.1 N NaOH per 100 cc. of milk. Owing to the fact that the slope of a titration curve of milk is

not steep through the pH range in which phenolphthalein undergoes its color change, the titers cannot be considered as accurate, but the following results show how great was the change in "titratable acidity."

	Control.	Proteus culture.
Titration value	26	43
H ion concentration	34×10^{-8}	2×10^{-8}

If we consider a hydrogen ion concentration of 10×10^{-8} (1×10^{-7}) to be that of a neutral solution, the uninoculated milk was distinctly acid while the milk which was acted upon by *B. proteus* became distinctly alkaline; yet the "titratable acidity" (to phenolphthalein) increased greatly. This increase was not "in spite of" but *because of* the hydrolysis of the protein and the consequent increase in the buffer action against added alkali. This phenomenon has been encountered so frequently in studies of proteolysis that no detailed proof need be given here.

While hydrolysis of a protein will always increase the buffer action of a solution in some particular pH region, it does not necessarily follow that the hydrogen ion concentration will be changed in one direction. This will depend upon the relative values of the acidic and basic dissociation constants of the amphoteric bodies set free and in culture media the effect will be superimposed upon that of the simple acids or bases which are formed.

The resultant effect would be very difficult to analyze and we shall not attempt to do so in this case. The point we do emphasize is that theoretically and experimentally an increase in "titratable acidity" is not incompatible with a decrease in hydrogen ion concentration or *vice versa*. The two phenomena should not be confused.

Before leaving this digression and returning to our main topic, we may again note as we did in our earlier paper that the general principles which we have used in developing our differential test may find a variety of applications. Possibly their application to the Smith reaction (Smith, 1905, 1910) for distinguishing human from bovine tubercle bacilli might explain some of the difficulties which have attended its use or at least clarify some of those difficulties in its study which have been mentioned by Harden (1913).

The Chemical Basis for the Division of Coli-Aerogenes Bacteria into Two Groups.

By independent routes we have now arrived at a point where it is profitable to review briefly a few of the chemical means for the separation of coli-aerogenes bacteria into two distinct groups.

Through the researches of Harden (1901), Harden and Walpole (1906), Thompson (1911), and others, it has been found that one of the chief distinctions between the products of the fermentation of dextrose by *B. coli* on the one hand and by *B. aerogenes* and *B. cloacæ* on the other is a quantitative one. In each case there are formed lactic, succinic, acetic, and formic acids and ethyl alcohol, but the proportions of these found in coli cultures are distinct from the proportions found in aerogenes and cloacæ cultures. These differences might have become the basis for a differential test and indeed were so used by Harden (1905); but the analytical difficulties involved prevented the extensive use of this method.

The quantitative differences in gas production by these organisms were recognized by Smith (1893) and upon these differences Smith established the fermentation tube test. Because the characteristics studied differ only in a quantitative way while the method involves sources of serious analytical error, the Smith fermentation tube has been of limited usefulness. Harden (1899), Harden and Walpole (1906), and Thompson (1911) established somewhat more accurately the gas ratios, but it remained for Keyes (1909) and Keyes and Gillespie (1912) not only to furnish reliable data for *B. coli* but to frame the method which with slight modifications has proved to be so useful in the more extensive investigations by Rogers and his collaborators. In these later researches (Rogers, Clark, and Davis, 1914; Rogers, Clark, and Evans, 1914, 1915) the value of analytical accuracy in the determination of the gas ratios was firmly established.

In their quantitative studies Harden and Walpole (1906) were seeking for the carbon deficit among the products of the fermentation of dextrose by *B. aerogenes* and encountered a butylene glycol and acetyl methyl carbinol. The latter compound was shown by Harden (1906) to be responsible for the reaction of Voges and Proskauer (1898). *B. cloacæ* (Thompson, 1911) produces the same substances, but these products are not formed by *B. coli*. Here we have a qualitative difference and the establishment on a firm basis of another cultural test.

Clark (1915), starting from the point of view established by Michaelis and Marcora (1912), attacked the problem of acid production of *B. coli* in a manner very different from that of Harden, and Clark and Lubs (1915) devised the differential test the revision of which is described in this article.

We have now the three tests: the gas ratio, the Voges-Proskauer reaction, and the indicator test of Clark and Lubs. The

correlation between the first and second was established on a limited scale by the researches of Harden, Walpole, and Thompson. On a more extensive scale, the correlation between the first and third was established by Clark and Lubs and between the second and third by Winslow and Kligler, Levine, Hulton, Greenfield, and Johnson, in 1916. Lastly the correlation among all the three tests is shown in this article.

It is suggestive to note that the establishment of firm bases for all three tests was the result of quantitative researches. While it is true that the Voges-Proskauer reaction as ordinarily used is a qualitative test its explanation was the result of some quantitative researches and its present defect lies in its qualitative nature as already noted.

The three tests correlate most beautifully in dividing the coli-aerogenes bacteria into two main groups. Within these groups there are doubtless distinct species or varieties, but the attempt to make any very sharp subdivision upon chemical evidence would be at present premature.

The three tests have been useful in showing that the two groups have in the main different habitats and it is suggestive to reverse the view of this relationship and to note that the conditions of these diverse habitats favor one or the other of the two types. Thus there is good reason to believe that two distinct natural groups have been differentiated.

There are other tests and correlations which might be mentioned were there need of further evidence that there is a clear and easily detected difference between two groups in the colon-aerogenes family. For instance, it has been found in the work conducted here that there is a very good correlation between a positive indol test and a low gas ratio, positive methyl red test and negative Voges-Proskauer reaction. Winslow and Kligler (1916) confirmed this with their series of cultures. But our experience has shown that the indol test has not yielded the perfect correlation obtained with the other tests, and for this reason as well as because the production of indol has not as yet been satisfactorily investigated we are inclined to assign to it a position of secondary importance.

It will be clearly recognized that the gas ratio, the production of acetyl methyl carbinol, and the phenomena made use of in the

methyl red test are not singly or collectively specific tests for *B. coli* or *B. aerogenes*. There are abundant indications in the literature that gas ratios similar to if not identical with those furnished by coli or aerogenes cultures are furnished by organisms of different types. Acetyl methyl carbinol has been found among the products of fermentation of several bacteria as noted in the papers by Harden and his collaborators and in more recent articles. Its occurrence in vinegar has recently been confirmed by Balcom (1917). A limiting zone of hydrogen ion concentration similar to that reached by coli cultures will doubtless be found for a variety of organisms. A reversion of reaction such as is made use of in the methyl red test is a common phenomenon.

Thus the tests which have been discussed must be considered as *differential* and may be applied only when the organism in question has been placed in the coli-aerogenes family. A definition of the boundaries of this family does not lie within the province of this paper, but it is permissible to mention a limitation imposed upon the differential tests by a commonly accepted definition of a salient cultural characteristic of the members of this family of bacteria.

It is commonly held that an organism to be a member of the colon-aerogenes family must ferment lactose. We shall not discuss the doubtful propriety of requiring gas production or acid production or both as the criterion of lactose *utilization*, but we do wish to emphasize the following considerations.

Having chosen for study organisms which we consider to be lactose fermenters we find that the application of the differential tests separates the series into two groups. With this separation as a basis, some sort of order is obtained in the correlation of a variety of other tests, and the correlation with sources becomes most striking and significant. The result is a much more suggestive and valuable differentiation than that obtained by the McConkey scheme, but there remains this serious consideration. If a fundamental cultural requirement of a member of the colon-aerogenes family is that it shall ferment lactose, there is imposed the same sort of requirement for the characterization of a whole family as is imposed by the McConkey scheme when groups within the family are separated on the basis of the fermentation of another sugar, sucrose.

The fermentation of sucrose has been found to be far less useful than the differential tests we have mentioned for the separation of groups within the family. May not the same objection be urged against the use of lactose as a salient and fundamental requirement for the whole coli-aerogenes family?

It may be urged that such a proposition is academic, that practical experience has shown the fermentation of lactose to be a convenient and significant crucial test, and that an overlapping of species is to be expected and must be treated biometrically with certain arbitrary points of reference.

Such arguments may be allowed whatever force they possess. They do not invalidate the fact that sucrose fermentation has been shown to be less valuable than the other differential tests nor the argument that since this is so the fermentation of lactose *may* prove to be less useful than other tests.

What these other tests would be we do not know, but if further researches conducted upon a broad and accurate basis should reveal the use of lactose to be as arbitrary as the use of sucrose fermentation, then it may become necessary to reconstruct the definition of the colon-aerogenes family. What position the differential tests we have discussed will then take cannot be foretold, and until further investigations reveal the position of these tests in larger schemes of classification there will remain some doubt of the permanent value of such schemes of classification as the provisional one suggested by Levine (1916, *c*).

Realizing that knowledge of the conduct of organisms termed for convenience *B. coli* or *B. aerogenes* is still fragmentary and that there are broader issues for the future than the mere differentiation of cultures within an arbitrary group, Rogers has been reticent in ascribing names or other than temporarily convenient schemes of classification to the extensive collection of organisms studied in this Division. We agree with him that questions of taxonomy must remain for some time to come entirely subordinate to careful and extensive surveys with quantitative methods upon the metabolism, the conditions of metabolism, and the distribution of various bacteria.

Therefore, while the differential tests which we have described may be of immediate practical value in routine analyses, we hope that their position in the larger scheme of classification will

not be misjudged and that the principles upon which these tests are based will be subjected to further research and not be abused by those whose only inspiration is to vary a test at will to see what happens.

Several of the quantitative aspects of the conduct of coli-aerogenes bacteria in the medium we have suggested are worthy of far more extensive investigation than we have had the time to give; but in view of the fact that the differential test may be a valuable one for field use within the immediate future the direction of such further research as may become necessary had best be determined by the unforseen difficulties which may arise in the practical examination of contaminated material and especially of the water supplies of armies in the field.

SUMMARY.

In studies of bacteria of the coli-aerogenes family, Rogers, Clark, and their collaborators have found that the accurately determined ratios of the gases CO_2 and H_2 produced in the anaerobic fermentation of dextrose furnish a reliable basis for the separation of the family into two distinct groups. In these same studies there were established certain striking correlations between the gas ratios and the sources of the cultures. These correlations were seen to have considerable significance to the sanitarian and it became imperative to devise some test which would correlate perfectly with the gas ratios and which would be available for routine use. Such a test was found by Clark and Lubs who took advantage of certain differences in the intensities of the acid fermentations and developed a medium in which these differences could be made apparent by the use of the indicator method of determining hydrogen ion concentrations. The present paper deals chiefly with a revision of the medium first proposed.

The new medium is composed of 0.7 per cent Na_2HPO_4 (anhydrous), 0.2 per cent KH phthalate, 0.1 per cent aspartic acid, and 0.4 per cent dextrose. The main principles used in designing this medium are described.

It was found that low gas ratio cultures (the type occurring in feces) in the new medium as in the old medium become red to methyl red, while high gas ratio cultures are yellow.

One hundred and sixty-four cultures which were classified definitely as low ratio cultures by gas analysis were all colored red by methyl red.

Sixty-five cultures which were classified definitely as high ratio cultures, the type occurring in grain, by gas analysis were all colored yellow by methyl red.

Including those cultures which were definitely classified by gas analysis and tested in the old medium but not in the new, there is now a total of 374 cases in which there has been a perfect correlation between gas ratios and the methyl red test.

Sixteen cultures studied in the present series were considered "atypical" because of abnormalities in gas production. The results with these organisms are tabulated but not discussed for lack of sufficient data.

The revised medium was found to be more suitable for making the Voges-Proskauer test than media formerly employed in this laboratory. In several cases positive reactions were indistinct and fleeting, but by careful observations a perfect correlation between the Voges-Proskauer tests and the methyl red and gas ratio tests was found in all typical cultures.

The reversion of reaction which is made use of in the methyl red test is discussed and it is shown that it cannot be attributed to ammonia production alone.

Possible applications of the principles involved in the methyl red test are suggested and the position of the test with its correlated tests in larger schemes of classification is discussed.

BIBLIOGRAPHY.

Ayers, S. H., and Rupp, P., 1915, *Science*, xlii, 318.
Balcom, R. W., 1917, *J. Am. Chem. Soc.*, xxxix, 309.
Clark, W. M., 1915, *J. Biol. Chem.*, xxii, 87.
Clark, W. M., and Lubs, H. A., 1915, *J. Infect. Dis.*, xvii, 160.
Clark and Lubs, 1916, *J. Biol. Chem.*, xxv, 479.
Clark and Lubs, 1917, *a*, *J. Bacteriol.*, ii, 1, 109,191.
Clark and Lubs, 1917, *b*, *J. Agric. Research*, 1917, x (July, in press).
Cole, S. W., and Onslow, H., 1916, *Lancet*, cxci, 1011.
Dodge, F. D., 1915, *J. Ind. and Eng. Chem.*, vii, 29.
Faraday, M., 1839, Experimental Researches in Electricity, London, i, note, p. 191.
Greenfield, M., 1916, *J. Infect. Dis.*, xix, 647.

Harden, A., 1899, *Tr. Jenner Institute*, Series 2, ii, 126.
Harden, 1901, *J. Chem. Soc.*, lxxix, 610.
Harden, 1905, *J. Hyg.*, v, 488.
Harden, 1905–06, *Proc. Roy. Soc., Series B*, lxxvii, 424.
Harden, 1913, Final Report of the Royal Commission on Human and Animal Tuberculosis, London, Part II, Appendix, vol. vi.
Harden, A., and Walpole, G. S., 1906, *Proc. Roy. Soc., Series B*, lxxvii, 399.
Harden, A., and Norris, D., 1911, *J. Physiol.*, xlii, 332.
Harden and Norris, 1912, *Proc. Roy. Soc., Series B*, lxxxiv, 492.
Hudson, C. S., and Dale, J. K., 1917, *J. Am. Chem. Soc.*, xxxix, 320.
Hulton, F., 1916, *J. Infect. Dis.*, xix, 606.
Johnson, B. R., 1916, *J. Bacteriol.*, i, 96.
Kendall, A. I., Day, A. A., and Walker, A. W., *J. Am. Chem. Soc.*, 1914, xxxvi, 1937.
Keyes, F. G., 1909, *J. Med. Research*, xxi, 69.
Keyes, F. G., and Gillespie, L. J., 1912–13, *J. Biol. Chem.*, xiii, 305.
Kligler, I. J., 1916, *J. Bacteriol.*, i, 663.
Levine, M., 1916, *a*, *J. Infect. Dis.*, xviii, 358.
Levine, 1916, *b*, *J. Bacteriol.*, i, 153.
Levine, 1916, *c*, *J. Bacteriol.*, i, 619.
Michaelis, L., and Marcora, F., 1912, *Z. Immunitätsforsch., Orig.*, xiv, 170.
Rogers, L. A., Clark, W. M., and Davis, B. J., 1914, *J. Infect. Dis.*, xiv, 411.
Rogers, L. A., Clark, W. M., and Evans, A. C., 1914, *J. Infect. Dis.*, xv, 100.
Rogers, Clark, and Evans, 1915, *J. Infect. Dis.*, xvii, 137.
Rogers, L. A., Clark, W. M., and Lubs, H. A., 1916, *J. Bacteriol.*, i, 82.
Schiff, H., 1884, *Ber. chem. Ges.*, xvii, 2929.
Sörensen, S. P. L., 1909, *Compt. rend. trav. Lab. Carlsberg*, viii, 1.
Smith, T., 1893, Wilder Quanter-Century Book, 187.
Smith, 1904–05, *J. Med. Research*, xiii, 253, 405.
Smith, 1910, *J. Med. Research*, xxiii, 185.
Thompson, J., 1911, *Proc. Roy. Soc., Series B*, lxxxiv, 501.
Thompson, 1912–13, *Proc. Roy. Soc., Series B*, lxxxvi, 1.
Voges, O., and Proskauer, B., 1898, *Z. Hyg.*, xxviii, 20.
Walpole, G. S., 1910–11, *Proc. Roy. Soc., Series B*, lxxxiii, 272.
Winslow, C.-E. A., and Kligler, I. J., 1916, *J. Bacteriol.*, i, 81.

THE THERMAL DECOMPOSITION OF THE OXYTOCIC PRINCIPLE OF PITUITARY SOLUTION.

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The evaluation of extracts of the posterior lobe of the pituitary depends on the presence in such extracts of a substance or substances which in very high dilution stimulates isolated uterus preparations. This oxytocic principle is ordinarily thermostable, and methods of manufacture generally involve boiling to remove coagulable material, and subsequent sterilization by heat. During certain experimental work on pituitary solution there was produced an extract of which the oxytocic principle was rapidly destroyed by boiling. This led to the following study of the rate and manner of the thermal decomposition of the principle, and some of the factors which condition it. The quantitative study of the progress of a reaction furnishes a method of attack which has added greatly to our knowledge of the properties of substances on which, because of their instability or occurrence in minute proportions, the usual chemical methods can throw but little light.[1]

The pituitary solution under investigation had been made from the posterior lobes of the pituitary glands of cattle. These were finely ground with sand, and extracted with $\frac{N}{60}$ acetic acid. The solution was boiled and filtered, and a few drops of chloroform were added. The solution was acid to litmus, and gave a brown color with alizarin and a yellow with methyl orange, so that the H ion concentration was of the order of $N \times 10^{-5}$.

[1] S. Arrhenius (Quantitative Laws in Biological Chemistry, London, 1915) gives a series of examples of the application of such methods to biochemical problems.

Qualitative experiments showed that heating rapidly diminished its activity.

The rate of decomposition was studied quantitatively at 100°C. This temperature was chosen because it is involved in the manufacture of pituitary extract, and because at this temperature the decomposition takes place conveniently rapidly. A flask of 150 cc. capacity served as the reaction vessel. It was immersed in a bath of boiling water, and errors introduced by variations in barometric pressure or traces of dissolved substances were disregarded as within the limits of experimental error of the assay method. A reflux condenser prevented changes in concentration of the extract due to evaporation. A small hole blown in the upper side of the flask and closed with a rubber plug permitted the withdrawal of samples from time to time by a capillary pipette. Since it seemed possible that the decomposition involved oxidation, air was excluded from the reaction flask by mean of a stream of carbon dioxide, which was carried in by a small tube passing down the condenser tube.

After the reaction flask had been brought to 100° a 50 cc. sample of the extract was introduced through the small hole in the side. At definite intervals a sample of about 5 cc. was removed, sealed off in a test-tube, and cooled at once to 0°, after which it was placed in the ice box to be assayed as soon as possible. The extract was at all times handled under aseptic conditions.

The samples were assayed by the isolated uterus method elaborated by Roth.[2] A slight modification was introduced in that the final dilutions were made directly in the suspension chamber, which was made up to a definite volume. As standard was used a 1:20,000,000 solution of β-iminazolylethylamine hydrochloride. The absolute concentration of the substance in decomposition being of course unknown, it becomes necessary to choose an arbitrary measure. The concentration of the active principle is accordingly expressed as the reciprocal of the dilution at which the extract is equal to the standard.

Table I gives the results of this experiment, the first column the time in minutes from the beginning of the experiment, the second column the strengths of the various samples, determined

[2] Roth, G. B., *Bull. Hyg. Lab., U. S. P. H., 100*, 1914.

TABLE I.

Decomposition of Pituitary Solution, 100°C., Air Absent, H Ion Concentration Approximately $N \times 10^{-5}$.

Time elapsed.	Concentration.		$K = \frac{1}{t} \log \frac{A}{A-x}$
	Observed.	Calculated.	
min.			
0	14,000	14,000	
10	10,000	10,312	0.03365
20	7,500	7,596	0.03121
35	5,000	4,802	0.02948
55	3,000	2,605	0.02802
Average.....			0.03058

physiologically and expressed in arbitrary units. Plotting the results showed that the concentration falls essentially as a logarithmic function of the time, and the reaction is apparently one of the first order. The last column accordingly represents the constant calculated from the formula for a monomolecular reaction, while in the third column are given the concentrations calculated from the same formula by substituting the average value of K. The agreement between observed and calculated values must be looked upon as satisfactory, particularly as the method employed is subject to the limitations in accuracy inherent in physiological determinations.

From these results follows the important conclusion that one is here observing the decomposition of a single substance. If more than one substance is present which to any significant extent affects the isolated uterus in the concentrations studied, the facts demand the difficult assumption that the two or more active substances are all decomposing at essentially the same rate. An attempt to follow the decomposition below the dilutions given gives rise to exceedingly irregular results. Several factors may contribute to this. One is the possibility of other constituents of pituitary extract, which are affected less or not at all by heating and which in these relatively high concentrations may affect the test organ. In not too high dilutions tissue extracts in general have the property of stimulating the isolated uterus. A further factor, to which the foregoing perhaps contributes, and

to which attention has recently been called,[3] is the increasing inaccuracy of the assay method as the dilutions become low. The slight progressive fall in the constant, if one may attach any significance to it, indicates the presence of a second substance, little effective at the higher dilutions, but gradually coming into evidence at the increasing concentrations which must be used. The fact remains that the oxytocic value of the pituitary solution, as measured by the isolated uterus method, has fallen to nearly one-fifth of its original strength in a manner characteristic of a single substance in decomposition. It seems impossible to escape the conclusion that the physiological assay of pituitary solution by the isolated uterus method, in the strengths practically employed, consists essentially in the measurement of a single substance.

One of the most elaborate investigations of the posterior lobe of the pituitary, from the standpoint of its individual active principles, is that of Führer.[4] This investigator isolated four substances, all of which contribute to the general physiological effect of pituitary extract. These were investigated pharmacologically with respect to their action on blood pressure and respiration, and on the uterus muscle, both the isolated uterus and the organ *in situ* being used. In the action on the uterus only two of these fractions, Products III and IV, seem practically to be involved. Führer's data, unfortunately in the present connection, do not permit an exact comparison of the activity of these two on the isolated guinea pig uterus. On the rabbit uterus *in situ* both are about of the same activity. But on the isolated guinea pig uterus Product IV seems effective in rather higher dilutions.[5] Moreover, this fraction seems to be present in considerably larger amounts than is Product III, and in spite of its activity it is not a pure substance. It seems possible, accordingly, that the assay of pituitary extract by the isolated uterus method consists in the measurement of a single substance which is a constituent of Führer's Product IV. The significance of this pos-

[3] Hamilton, H. C., and Rowe, L. W., *J. Lab. and Clin. Med.*, 1916-17, ii, 120.

[4] Führer, H., *Z. ges. exp. Med.*, 1913, i, 397.

[5] Führer,[4] Figs. 23 and 24, pp. 427 and 428.

sibility lies in the fact that the characteristic effect of pituitary extract on the blood pressure is practically lacking in Product IV.

The isolated uterus method of evaluating pituitary solution has recently been subjected to criticism by Hamilton and Rowe.[3] Objection was raised on two grounds; first, that action on the isolated uterus need not indicate clinical oxytocic value; and second, that action on the isolated uterus need not be a measure of blood pressure effect.

The first of these objections seems not altogether well grounded. Fühner's results indicate clearly that in pituitary extract the same substances that affect the isolated uterus also stimulate this organ *in situ*. In the present investigation it was possible by heating to destroy the action on the isolated uterus. Pituitary extract of which this activity had thus been destroyed was subjected to clinical observation. These observations were naturally limited, but altogether definite; they indicate that with the destruction of the action on the isolated uterus disappears also the property of stimulating the uterus in labor. There is no evidence that the one property is ever present without the other in pituitary extract, and so far as its use in obstetrics is concerned, proof is wanting that the isolated uterus does not furnish an effective method of evaluation.

With the blood pressure effect of pituitary extract the case may be different. Fühner's results have shown definitely that the pressor effect and the oxytocic effect need not be due to an identical substance. Herring[6] found that pars intermedia extracts, while acting on the uterus, had no specific effect on the blood pressure. Further definite evidence is brought forward in Roth's[7] recent paper, in which certain extracts were found equal by one method, while by the other method one was double the strength of the other. The results at hand indicate that the official assay method may involve the measurement only of a substance in no wise concerned with the pressor effect. But if these results argue against the isolated uterus method of evaluating pituitary extract which is to be used for its pressor effect, they argue conversely against the blood pressure determination as a

[6] Herring, P. T., *Quart. J. Exp. Physiol.*, 1914, viii, 267.
[7] Roth, *Bull. Hyg. Lab., U. S. P. H., 109*, 1916.

measure of oxytocic activity. Definite light should be thrown on this question by following quantitatively the pressor activity while the oxytocic principle is being destroyed by heat, as in the experiment described. A limited number of preliminary observations along this line do not indicate that under these conditions the two properties are affected in qualitatively different ways. No doubt in the practical preparation of pituitary extract the two properties may go hand in hand. But it is safe to say that at present pituitary extract can be evaluated absolutely rationally only by testing it with respect to the specific effect for which it is to be employed.

It has been suggested by Fenger[8] that the active principle is susceptible to oxidation by the air. The rapid deterioration of the extract here described in an atmosphere of carbon dioxide made it seem improbable that the presence of oxygen would greatly influence the reaction. The experiment was, however, repeated, using the same extract, but substituting a current of air for the carbon dioxide previously used. The extract had lost greatly in strength, even at ice box temperature, in the 10 or 12 days that had intervened, and only two observations were possible before concentrations were reached at which the results could not be followed significantly. Table II gives the figures obtained. The calculated value is arrived at by substituting the average constant from the preceding experiment, 0.03058.

TABLE II.

Decomposition of Pituitary Solution, 100°C., Air Present, H Ion Concentration Approximately $N \times 10^{-5}$.

Time elapsed.	Concentration.		$K = \frac{1}{t} \log \frac{A}{A-x}$
	Observed.	Calculated.	
min.			
0	4,600	4,600	
10	3,375	3,387	0.03097

The reaction has evidently not been influenced in any significant manner by the presence of oxygen. Attention was accordingly turned to another possible factor, the H ion concentration

[8] Fenger, F., *J. Biol. Chem.*, 1916, xxv, 417.

of the solution. This proved to be the essential factor. The H ion concentration of the unstable solution had been of the order of $N \times 10^{-5}$. If now the free acidity be increased until the solution is just acid to methyl orange but still gives a violet color with methyl violet—so that the H ion concentration is of the order of $N \times 10^{-3}$—the oxytocic principle becomes thermostable. Such a solution may be heated to 100°C. for an hour without demonstrable change, as the following typical experiment shows.

TABLE III.

Stability of Pituitary Solution, 100°C., Air Present, H Ion Concentration Approximately $N \times 10^{-3}$.

Time elapsed.	Concentration observed.
min.	
0	20,000
25	20,000
55	20,000

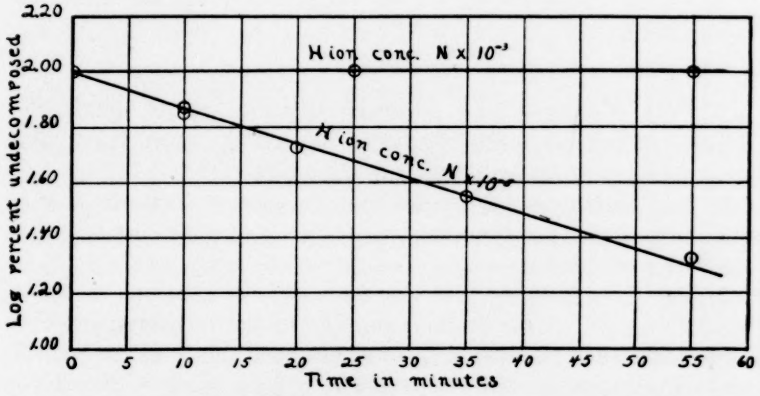

FIG. 1. The chart represents graphically the results of the three experiments, and shows that decomposition proceeds as a logarithmic function of the time. To bring the experiments to a common scale, the percentage of the total active substance remaining undecomposed is substituted for the concentration.

If we may assume that the oxytocic principle is a complex amine, for which there exists certain chemical and pharmaco-

logical evidence,[9] the results at hand are able to throw some light on its chemical characteristics. In acid solution it must exist as a salt, fully ionized at the high dilution. The free base present in alkaline solution must yield, so far as it is ionised, the same cation as the salt. It is to be supposed, therefore, that the cation is stable, and that it is the free base which decomposes. No doubt the base is relatively weak, and the salt subject to hydrolysis. A decrease in the acidity of the solution results then in increasing the concentration of free base, and correspondingly hastening the decomposition. The possibility that the free base rearranges to a physiologically inert compound suggests itself, and is not without analogy among organic compounds. The rearrangement is irreversible or the reversal involves a time reaction, since activity is not restored by making the solution again acid.

Attention has been called to the fact[8] that dilutions of pituitary extract in Locke's solution deteriorate rapidly, a circumstance no doubt explained by the low H ion concentration of the solution. These facts must also have their bearing on the stability of the active principle in various body fluids, and the duration of its effect.

SUMMARY.

1. That constituent of pituitary solution which affects the isolated uterus is rapidly destroyed at 100°C. when the H ion concentration of the solution is of the order of $N \times 10^{-5}$.

2. The destruction proceeds in a manner characteristic of a single substance decomposing according to the law for a monomolecular reaction.

3. The facts indicate that the evaluation of pituitary solution by the isolated uterus method consists in the measurement of a single substance. There is no evidence that this does not constitute an adequate measure of clinical oxytocic value. The possibility is suggested that the substance measured may not be concerned in the characteristic pressor effect of pituitary solution.

4. Oxygen seems not to be involved in the decomposition under the conditions of the experiment.

5. The oxytocic principle becomes thermostable when the H ion concentration is increased to the order of $N \times 10^{-3}$.

[9] Führer, *Münch. med. Woch.*, 1912, lix, 852. Aldrich, T. B., *J. Am. Chem. Soc.*, 1915, xxxvii, 203.

NUTRITION INVESTIGATIONS UPON COTTONSEED MEAL. II.

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In an earlier paper we have reported the results of nutrition investigations upon the efficiency of cottonseed meal as a food for the albino rat. In this first report we supported certain conclusions regarding the efficiency of cottonseed meal in promoting the growth and development of the albino rat which have been more definitely substantiated during succeeding months of investigation. The results of several experiments on growth and reproduction, indicated at that time, together with additional data bearing upon our first conclusions will be considered in this paper.

The general plan and technique of the investigation previously described[1] has been followed in this latter work. In all the experimental diets the Allison cottonseed flour[2] has been employed exclusively. By a constantly improving process of bolting, the flour is deprived of the greater part of the lint, hulls, and resin contained in the ordinary commercial cottonseed meal and becomes a highly edible flour of the following composition:[3]

	per cent
Protein	51.19
Fat	11.40
Crude fiber	3.05
Nitrogen-free extract	22.22
Water	6.14
Ash	6.00

[1] Richardson, A. E., and Green, H. S., *J. Biol. Chem.*, 1916, xxv, 307.

[2] Allison flour is the trade name for the refined cottonseed flour manufactured by G. A. Baumgarten, Schulenburg Oil Mills, Schulenburg, Texas.

[3] Analysis made by G. S. Fraps, College Station, Texas, January, 1916.

In the experiments the following diets have been used:

	per cent
1. Cottonseed flour	50
Starch	22
Lard	28

	per cent
3A. Cottonseed flour	50
Starch	22
Lard	16
Butter fat	12

	per cent
4. Cottonseed flour	70
Lard	30

	per cent
7. Cottonseed flour	50
Starch	12
Lard	16
Butter fat	12
Protein-free milk[4]	10

	per cent
13. Cottonseed flour	45
Whole milk powder[5]	17
Starch	10
Lard	28

	per cent
14. Cottonseed flour	50
Protein-free milk	22
Lard	28

	per cent
16. Cottonseed flour	50
Casein	5
Lard	16
Butter fat	12
Starch	7
Protein-free milk	10

	per cent
17. Cottonseed flour	50
Casein	5
Lard	28
Protein-free milk	10
Starch	7

	per cent
18. Cottonseed flour	50
Casein	5
Lard	28
Starch	17

	per cent
19. Cottonseed flour	50
Casein	5
Butter fat	12
Lard	16
Starch	17

	per cent
21. Cottonseed flour	62
Butter fat	5
Lard	23
Protein-free milk	10

Can Animals Live on a Diet Containing at Least 50 per Cent Cottonseed Flour?

We have previously reported that animals have lived as long as 345 days on diets containing 33 to 50 per cent cottonseed flour. Since then rats have lived between 400 and 500 days

[4] Richardson and Green,[1] p. 309.

[5] Manufactured by Merrell-Soule, Syracuse, New York.

on 50 per cent cottonseed flour. One animal, Rat 40, lived to the age of 565 days on a diet containing 50 per cent cottonseed flour, even though deficient for a part of the time in butter fat[6] and mineral,[7] and later only mineral. The growth, development, and general well-being of these rats have depended upon the addition to the diet of certain necessary food substances other than protein.

Is the Protein of Cottonseed Flour Sufficient for the Normal Growth of the Albino Rat?

With diets in which the only protein is supplied by 50 per cent cottonseed flour rats have lived from 400 to 565 days. With 50 per cent cottonseed flour alone furnishing the protein, mineral, and growth-promoting factor, as in Diet 1, rats have lived as long as 270 days. The rate of growth has not been normal and had continued for a period ranging from 135 to 205 days (Chart 1). From this point if the animals are continued on Diet 1 they gradually lose weight and finally die, or they maintain themselves for a varying period before loss in weight and death occur. On a diet in which 70 per cent cottonseed flour furnishes the only protein, mineral, and growth-promoting factors, as in Diet 4, there has been normal growth and then slow continuous growth for 165 to 205 days (Chart 2). After this period the weight gradually declined. When the diet was changed so as to furnish 62 per cent cottonseed flour, 5 per cent butter fat, and 10 per cent protein-free milk, as in Diet 21, growth was resumed and there was a general improvement for 80 days longer.

When 12 per cent butter fat with its growth-promoting factor is added, as in Diet 3A, rats have lived as long as 287 days. The rate of growth with this addition to the diet is practically the same as without the butter fat, although as we first reported, with butter fat the period of growth is somewhat longer, ranging as high as 240 days of slow growth (Chart 3) before complete

[6] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913–14, xvi, 423.

[7] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxi, 615. Hart, E. B., McCollum, E. V., Steenbock, H., and Humphrey, G. C., *Wisconsin Agric. Exp. Station Research Bull. 17*, 1911. Osborne and Mendel, *J. Biol. Chem.*, 1913, xv, 311.

failure of the diet is demonstrated. On cottonseed flour as the only source of protein with the addition of the mineral of protein-free milk, but without butter fat as in Diet 14, there is no advantage in the growth of animals as compared with that of rats on Diets 1 and 3A (Chart 4).

A diet with cottonseed flour as the sole source of protein completely adequate for normal growth of the albino rat as compared with control animals is found in Diet 7, containing 50 per cent cottonseed flour but with the addition of both butter fat and the mineral of protein-free milk (Chart 5). From this chart it will be seen that this diet is adequate not only for normal growth of the rats reared from an early age but also supports normal growth of the second generation (Rat 1026).

With the idea of determining whether better growth could be induced by a combination of cottonseed flour with some protein which has been demonstrated efficient for growth, 5 per cent casein[8] was introduced as in Diet 16. From Chart 6 it will be seen that the addition of casein to this diet in the presence of both butter fat and protein-free milk is apparently no more favorable to growth than is Diet 7, similar in composition but without casein. While the addition of casein to a diet containing 50 per cent cottonseed flour, butter fat, but no protein-free milk, as in Diet 19, is apparently quite as efficient for growth as either Diet 16 or 7, Diet 18, containing 50 per cent cottonseed flour, 5 per cent casein, but no protein-free milk or butter fat, and Diet 17, similar to 18, but with the addition of protein-free milk, induce no better growth than similar diets, 1 and 14, without casein, as shown by Chart 7.

Is the Protein of Cottonseed Flour Sufficient for Normal Reproduction?

In the absence of either butter fat or protein-free milk, or of both, where cottonseed flour is the sole source of protein, as in Diets 1, 3A, and 14, no reproduction has been obtained, but reproduction to the third generation has been obtained with rats on Diet 7, containing 50 per cent cottonseed flour, 10 per cent protein-free milk, and 12 per cent butter fat. The first repro-

[8] Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 351; 1916, xxvi, 1.

duction on this diet was from Rat 38. This animal on Diet 3A, containing 50 per cent cottonseed flour, 12 per cent butter fat, but no additional mineral, grew normally for 145 days to the weight of 170 gm., which she maintained for 80 days with no reproduction although mated. When mineral in the form of protein-free milk was added, as in Diet 7, she immediately began to grow again and at the age of 13 months reproduced a family of seven, and 7 weeks later another family of six. All of the first litter, two males and five females, grew and developed normally to maturity (Chart 8) but only one of the females ever produced young. This rat, No. 1024, at the age of 6 months bore seven young. Only one of this litter, No. 2042, is alive at the age of 150 days.

Similarly, Rat 40, stunted successively for 192 days on Diet 1 and 35 days on Diet 3A, finally when transferred to Diet 7 began to grow steadily and at the age of almost 15 months produced a very weak litter of ten young, all of which died when 1 week old, and 3 months later bore a litter of about four, all of which either died or were eaten by the mother. Out of six families of a total of thirty-nine young borne by four females on Diet 7 only eight of the second generation have grown to maturity, and of seven young of the third generation only one animal has survived. An explanation of the high mortality of the third generation on this diet may be found in close breeding, as the parents of this third generation were of the same family.

Comparing in relation to reproduction Diet 16, containing 5 per cent casein, with Diet 7, there appear to be more favorable conditions for reproduction with Diet 16, for on this diet we have obtained frequent normal reproduction with a low rate of mortality as compared with Diet 7. On Diet 16 there have been three generations of rats. The first generation raised on Diet 16 since weaning was borne of Rat 125 which had been raised on Diet 13, containing 45 per cent cottonseed flour and 7.2 per cent milk protein (Chart 9).

Of eight families with a total of thirty-seven young borne by three females raised from weaning on Diet 16, twenty-eight have grown normally to maturity and three females of this second generation, Nos. 2008, 2016, and 2020, have produced three, five, and six young respectively.

On Diet 19, containing 5 per cent casein, 50 per cent cottonseed flour, 12 per cent butter fat, and no additional mineral, rats have grown practically normally, and one female, No. 240, has produced two litters of five and seven young, of which three females have survived (Chart 10). The ability of the animal body to grow and reproduce on this diet is particularly interesting when the ash analysis of the Allison flour is considered.

Ash Analysis of Cottonseed Flour (Golaz).

	per cent
Inorganic salts	5.5—6.0
SiO_2	0.14
Cl	0.00
SO_3	0.06
P_2O_5	2.57
K_2O	2.01
CaO	0.26
MgO	0.25
Na_2O	0.00

What Nutritive Factors Are Lacking in Cottonseed Flour?

As has been stated, Diet 7, containing 50 per cent cottonseed flour, 12 per cent butter fat, and 10 per cent protein-free milk, is adequate for normal growth, development, and reproduction of the albino rat as well as for the recovery to a normal condition of rats which have been stunted or reduced to an extreme degree of emaciation by diets deficient in some necessary food factors,[9] whereas it has been demonstrated that Diets 1, 3A, and 14, lacking either additional mineral, butter fat, or both, are not sufficient for either normal growth or reproduction. In the previous paper we gave no report as to whether this lack was due to deficiency in the inorganic salts or to the lack of some growth-promoting factor, but suggested that the results of our work indicated that a diet of 50 per cent cottonseed flour, lard, and starch lacks sufficient mineral and growth-promoting substances to induce normal growth. Additional light upon the question of the efficiency of the mineral of cottonseed flour for growth and reproduction has been obtained by a further consideration of diets containing casein. Diet 18, without either butter fat

[9] Richardson and Green,[1] p. 313.

or protein-free milk, resembling Diet 1 except in the addition of 5 per cent casein, and Diet 17, with 5 per cent casein but without butter fat, otherwise resembling Diet 14, induce no more normal growth than does either Diet 1 or 14. However, Diet 19 with 5 per cent casein but without protein-free milk, otherwise resembling Diet 3A, does induce normal growth as 3A does not. This indicates that cottonseed flour supplies enough mineral for normal growth when casein is added, which by its presence evidently increases the efficiency of a diet limited in its mineral content.

Is There Any Toxic Effect Due to the Feeding of Cottonseed Flour or Extracts in the Diet of the Albino Rat?

Our conclusions in regard to the supposed toxicity of cottonseed flour have been further substantiated by later work. In feeding as much as 70 per cent cottonseed flour as in Diet 4 during 209 days, and 50 per cent cottonseed flour during 565 days, there have been no harmful results apparent which could be attributed to toxic substances. Four successive generations of rats have been obtained on Diet 13, which contains 45 per cent cottonseed flour and 17 per cent milk powder, and three successive generations on Diet 16, the first lot of rats on this diet being the young of Rat 125, raised on Diet 13. The animals on Diets 13 and 16 were perfectly normal in growth and frequent reproduction.

To test further the question of the toxicity of cottonseed and cottonseed flour, extracts of both these substances have been fed.

Both petroleum ether and ethyl ether extracts from the entire cottonseed have been fed to rats. These extracts from the whole seed dried at 120°C. have been prepared according to the method described by Withers and Carruth.[10] The petroleum-ether-free extract, which consists largely of oil, is fed in the following diet:

	per cent
Whole milk powder	60
Starch	12
Lard	8–22
Petroleum ether extract	6–20

[10] Withers, W. A., and Carruth, F. E., *J. Agric. Research*, 1915–16, v, 267.

The ethyl-ether-free extract of the residue extracted first by petroleum ether which, as Withers says, amounts to about 2 per cent of the weight of the kernels used and therefore according to the same authority is about 40 to 50 per cent gossypol, is fed in the following diet:

	per cent
Whole milk powder	60
Starch	12
Lard	27.5
Ethyl ether extract	0.5

Computing upon the basis of the figures for gossypol content of the ethyl ether extract given by Withers, we suppose 1 gm. of this food to be equivalent to about 0.002 gm. of gossypol. Chart 12 gives the food intake and behavior of animals upon diets containing petroleum ether extract of the whole cottonseed, Lot A, and ethyl ether extract of the petroleum-ether-extracted cottonseed, Lot B.

The whole ether extract of Allison cottonseed flour, equivalent to about 8 per cent of the weight of the flour, has been fed in the following diet:

	per cent
Whole milk powder	60
Starch	12
Lard	18
Ether extract of flour	10

Chart 12, Lot C, indicates the food intake and behavior of rats on this diet.

Parallel with these experiments, recrystallized gossypol was fed.[11] The results of our work have corroborated the findings of Withers in regard to the toxic effect of gossypol. Rats which have received 0.4 per cent gossypol in a diet containing 60 per cent milk powder, 12 per cent starch, and 27.6 per cent lard have rapidly lost weight and died within 4 to 10 days, or when they had consumed 0.028 to 0.086 gm. of gossypol. When the amount of gossypol in the diet is decreased to 0.1 per cent, the food intake is greatly increased but the rats steadily lose weight. Rat 8 died after 21 days, having consumed 0.137 gm. of gossypol; Rat

[11] This was furnished by Dr. Withers, of the North Carolina Agricultural Experiment Station.

9, after 28 days, having consumed 0.221 gm. of gossypol, was changed to a diet containing 0.05 per cent gossypol, on which diet it made a slight gain in weight during 18 days, after which the weight has steadily declined (Chart 13).

SUMMARY.

1. Albino rats have lived for 400 to 565 days with 50 per cent cottonseed flour in the diet.

2. The protein in a diet containing 50 per cent cottonseed flour, protein-free milk, and butter fat is sufficient for normal growth and development of the albino rat and for reproduction to the third generation. No better growth is induced, but more frequent reproduction with lower mortality and more general well-being of animals are obtained by the addition of 5 per cent casein to a diet containing 50 per cent cottonseed flour, butter fat, and protein-free milk.

3. Normal growth and reproduction do not result from diets containing 50 per cent cottonseed flour in which there is a lack of butter fat, protein-free milk, or both. However, 50 per cent cottonseed flour with 5 per cent casein and butter fat, without additional mineral beside that furnished by the cottonseed flour, supports normal growth and reproduction although the second generation does not grow quite normally on this diet.

4. No toxic effect is apparent in feeding from 45 to 50 per cent cottonseed flour to albino rats through four successive generations or during 565 days of the life of an individual. Petroleum ether extract of the entire cottonseed fed in a well balanced diet has a depressing influence on the weight of the albino rat. Ethyl ether extract from petroleum-ether-extracted cottonseed and ethyl ether extract of Allison cottonseed flour have no harmful effect upon the albino rat.

The charts follow.

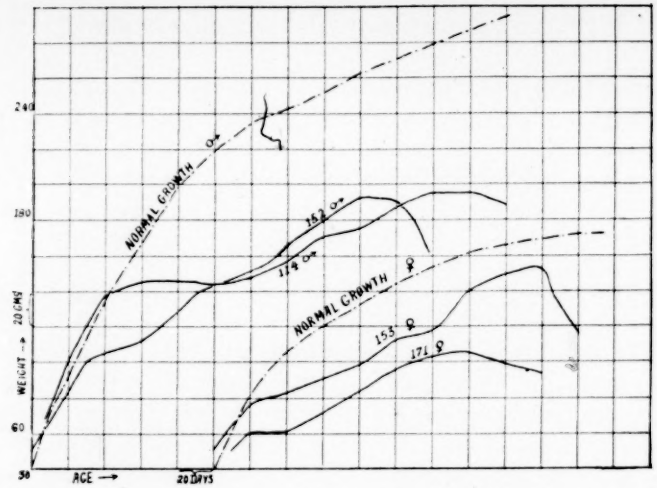

CHART 1. Rats do not grow normally on Diet 1, in which 50 per cent cottonseed flour furnishes the only protein, mineral, and growth-promoting factor. Growth on this diet has continued for 135 to 205 days.

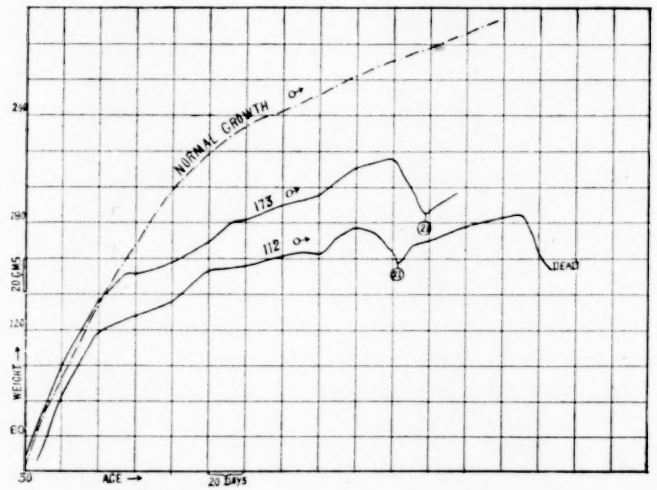

CHART 2. On Diet 4, in which 70 per cent cottonseed flour furnishes the only protein, mineral, and growth-promoting factors, rats grow normally for 40 days, and grow steadily but slowly for 165 to 205 days longer. From this point the weight gradually declines, but growth is resumed when the diet is changed so as to furnish 62 per cent cottonseed flour, 5 per cent butter fat, and 10 per cent protein-free milk, as in Diet 21.

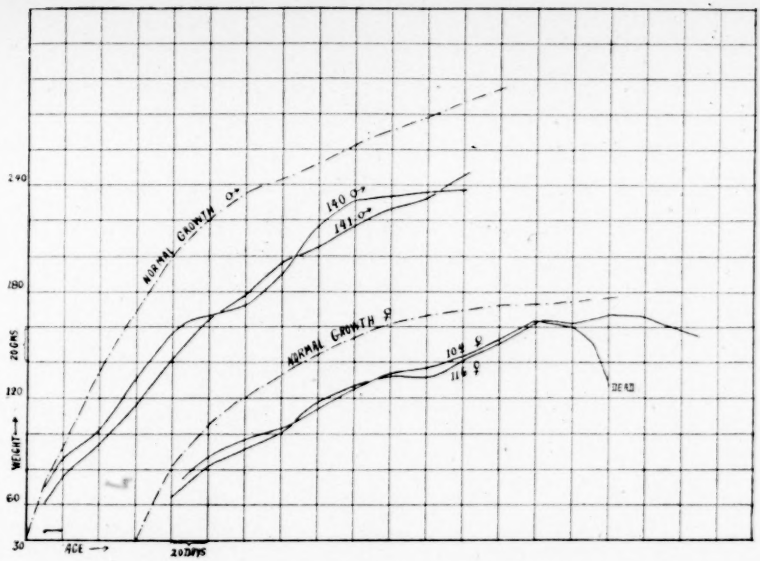

CHART 3. With the addition of butter fat, as in Diet 3A, there is practically no better rate of growth than without butter fat, as in Diet 1, but the period of growth is longer.

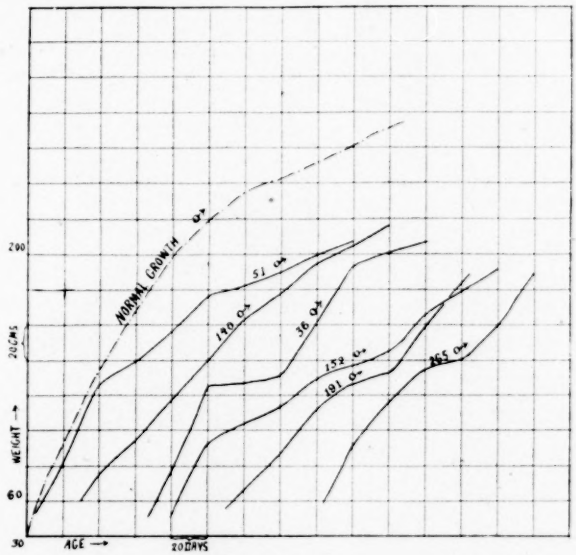

CHART 4. With the addition of the mineral in protein-free milk but without butter fat, as in Diet 14, there is no advantage in the growth of Rats 191 and 265, as compared with the growth of Rats 152 and 36 on Diet 1 and Rats 140 and 152 on Diet 3A.

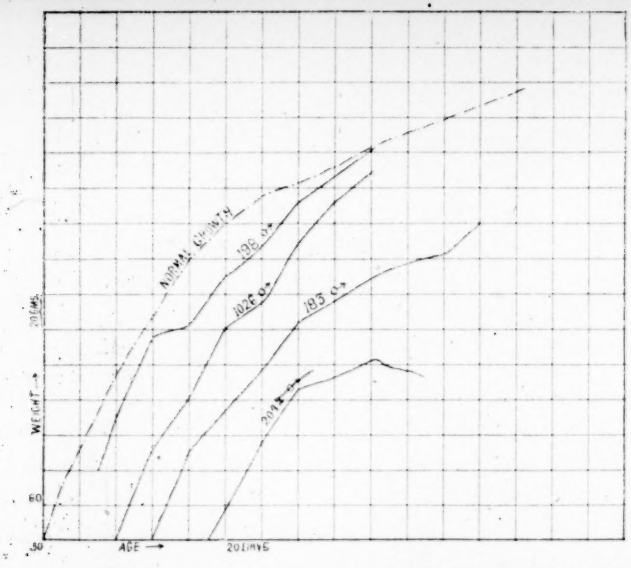

CHART 5. Diet 7, containing 12 per cent butter fat, 10 per cent protein-free milk, and 50 per cent cottonseed flour as the sole source of protein, is completely adequate for normal growth of rats reared from an early age, Nos. 198 and 183, and of the second generation, No. 1026, and supports normal growth of the third generation, No. 2042, for 50 days.

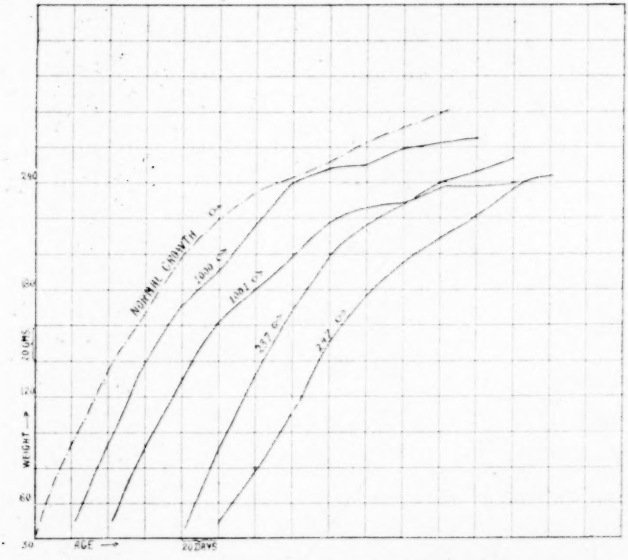

CHART 6. 5 per cent casein in a diet containing 50 per cent cottonseed flour, butter fat, and protein-free milk, as in Diet 16, is no more favorable to growth than Diet 7 without casein (Rats 1000 and 1001).

Diet 19, containing casein, cottonseed flour, butter fat, but no protein-free milk, is apparently quite as efficient for growth as either Diet 16 or 7 (Rats 237 and 247).

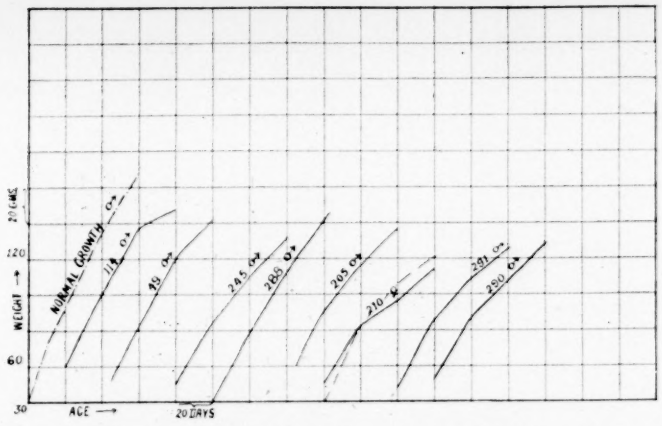

CHART 7. Rats 245 and 288 on Diet 18, containing 5 per cent casein and 50 per cent cottonseed flour but no protein-free milk or butter fat, grow no better than Rats 114 and 49 on Diet 1 without casein.

Rats 291 and 290 on Diet 17, containing 5 per cent casein, 50 per cent cottonseed flour, protein-free milk, but no butter fat, grow no better than Rats 205 and 210 on Diet 14 without casein.

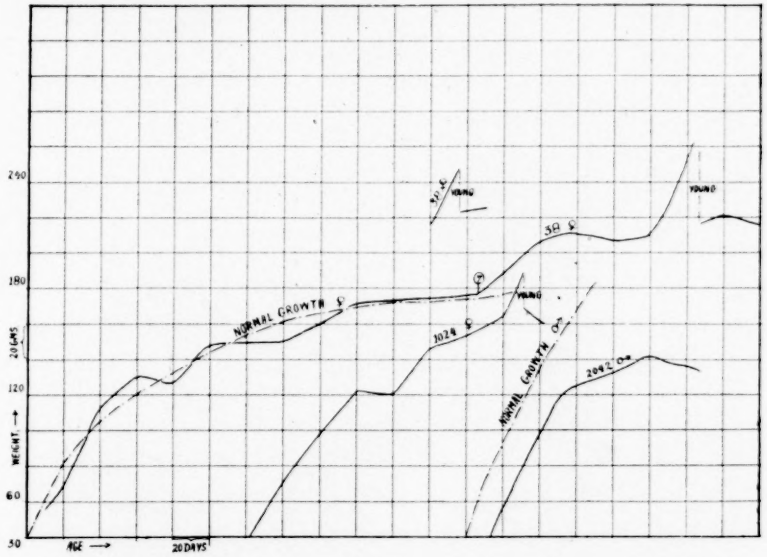

CHART 8. Rat 38 on Diet 3A did not reproduce when mated, but when transferred to Diet 7 she produced two families of young. Of the first litter, No. 1024 bore seven young. No. 2042 of the third generation is alive at the age of 150 days.

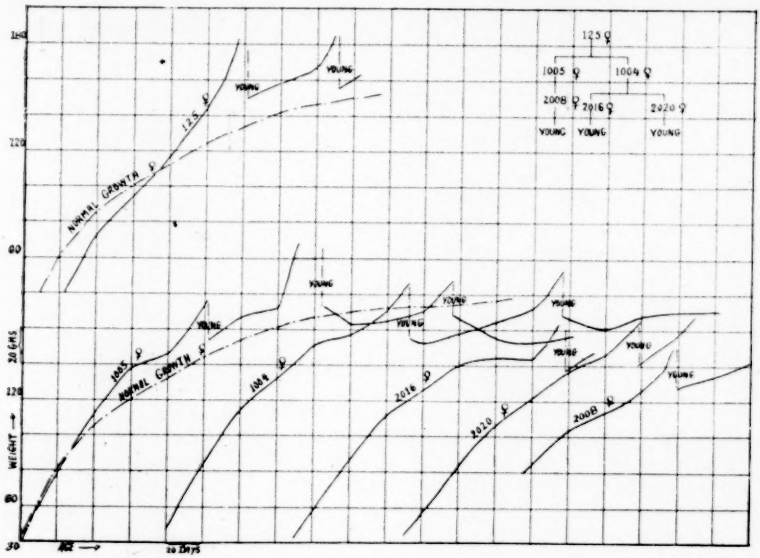

CHART 9. Three generations of rats have been obtained on Diet 16, the first generation having been obtained from Rat 125 raised on Diet 13.

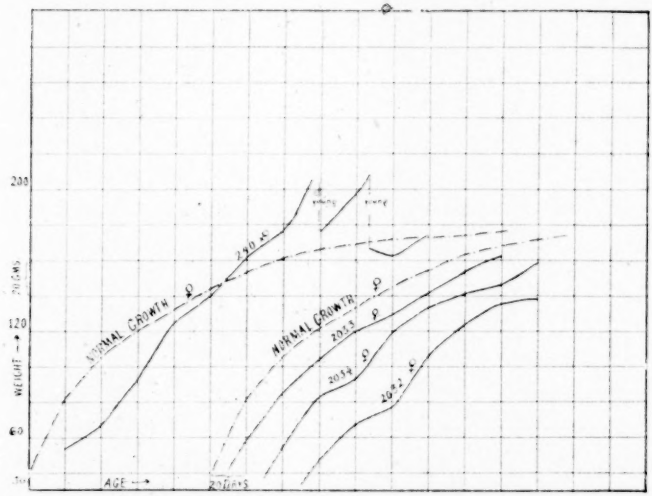

CHART 10. Rat 240 on Diet 19 has borne two litters. The second generation, Nos. 2032 and 2034, is not growing normally.

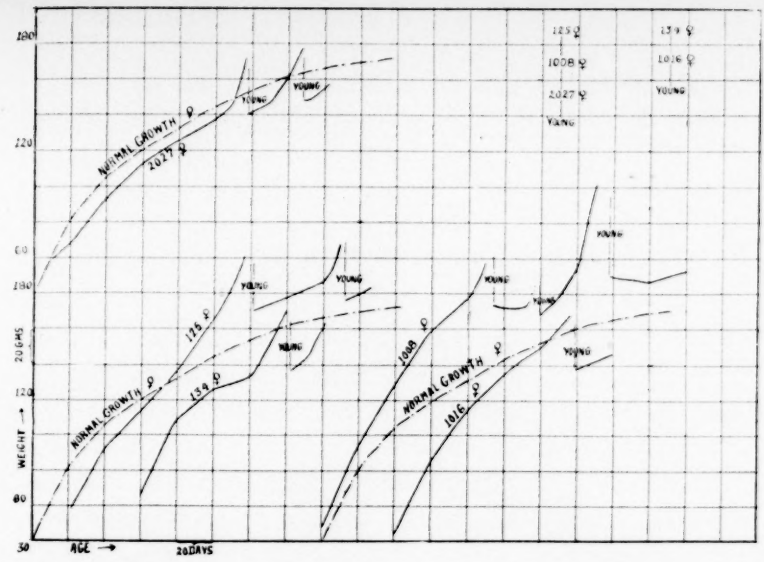

CHART 11. Four generations of rats on Diet 13, containing 45 per cent cottonseed flour.

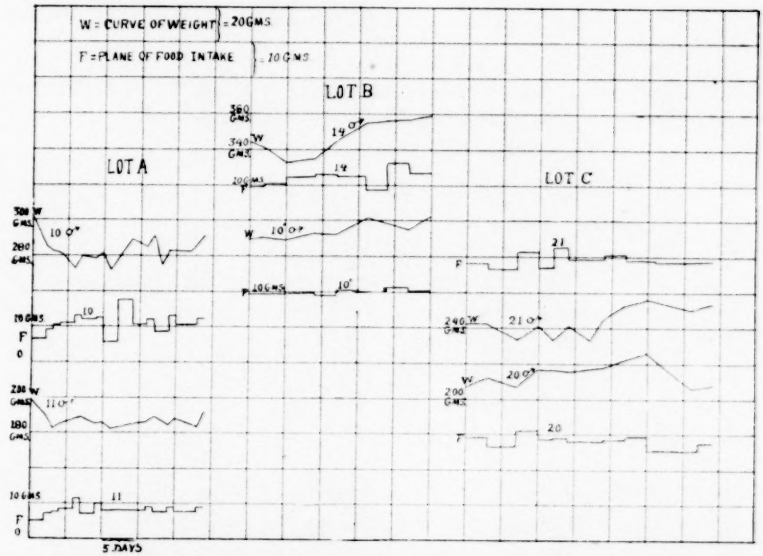

CHART 12. Lot A indicates the behavior and food intake of Rats 10 and 11 on a diet containing the petroleum ether extract of the entire cottonseed.

Lot B indicates the behavior and food intake of Rats 10 and 14 on a diet containing the ethyl ether extract of the petroleum-ether-extracted cottonseed.

Lot C. Rats 20 and 21 are on a diet containing the ether extract of Allison cottonseed flour.

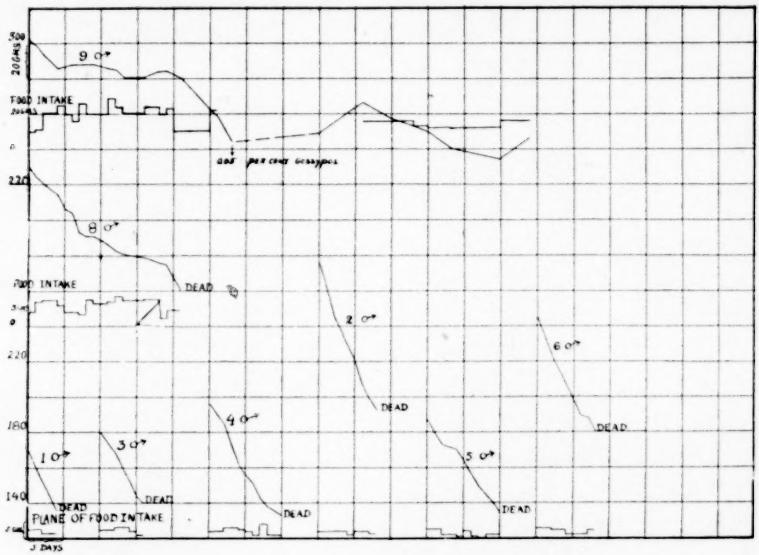

CHART 13. Rats 1, 2, 3, 4, 5, and 6 received 0.4 per cent gossypol. Rats 8 and 9 received 0.1 per cent gossypol for 21 and 28 days respectively. From this point Rat 9 received 0.05 per cent gossypol.

THE USE OF THE VAN SLYKE CO_2 APPARATUS FOR THE DETERMINATION OF TOTAL CO_2 IN SEA WATER.

By J. F. McCLENDON.

(*From the Physiological Laboratory of the University of Minnesota, Minneapolis.*)

(Received for publication, November 27, 1916.)

Since the apparatus for very accurate determinations of the CO_2 in aqueous fluids has usually been quite complicated and expensive, the present investigation was undertaken to determine the degree of accuracy that might be attained with Van Slyke's simplified mercury pump and gas burette, with special reference to sea water. With careful manipulation, an accuracy of 0.5 per cent was attained. In order to accomplish this, a large number of corrections had to be applied, and since these corrections vary so much for different determinations that they cannot be combined in a table such as Van Slyke has made for blood, an example of the results of a determination with the corrections applied is given below.

A solution of double normal HCl and one of half normal NaOH were prepared, to liberate and absorb the CO_2. The elementary gases have about the same solubilities in these solutions as in sea water, and the solutions were brought to equilibrium with the elementary gases at the same partial pressures as in sea water. In this way, any liberation or absorption of inert gas, due to mixing these solutions with sea water in the apparatus, was avoided.

The stock form of apparatus from Emil Greiner Company was used, but with a leveling bulb with perpendicular sides, in order to prevent an error in reading the height of the mercury. This cylindrical bulb was connected to the apparatus by means of a piece of vacuum tubing of soft rubber and of only 1 mm. bore, so as to reduce its weight and thus prevent interference

with vigorous shaking. The bulb was attached by means of a swivel joint to a screw of about 4 mm. pitch that was passed through a nut held in a burette clamp. By means of the screw, very accurate adjustment of level could be made.

The apparatus was cleaned with potassium bichromate in concentrated sulfuric acid, rinsed with distilled water, and the stop-cocks were greased with a preparation made by dissolving native rubber in boiling paraffin and adding vaseline in order to obtain the right consistency. The apparatus was filled with mercury in the usual manner, to about 2 mm. above the upper stop-cock, and evacuated once in order to remove air bubbles. It was refilled with mercury, care being taken that both capillary tubes above the upper stop-cock were filled. 10 cc. of sea water were measured in a pipette and introduced into the apparatus through the cup on top. In order to prevent error due to a drop of sea water remaining in the cup, only 1 cc. was admitted before the stop-cock was opened, and then the water was allowed to leave the cup as fast as it entered it. When the water level sank to the entrance of the capillary at the base of the cup, 1 cc. of double normal HCl was admitted, and the last drop of sea water washed into the apparatus with it. When the acid level sank to the entrance of the capillary, mercury was placed in the cup and allowed to pass into the apparatus, thus forcing in the last drop of acid. Care was taken that enough mercury was left in the two capillaries above the upper stop-cock in order to seal it. The apparatus was exhausted until the mercury reached the 50 cc. mark, the lower cock closed, and the apparatus inverted in order to see whether the upper stop-cock leaked. It was then held with the fingers far out at the extreme ends of the apparatus, in a horizontal position and shaken violently for 2 minutes or given 400 double vibrations, in order to bring the water into equilibrium with the reduced CO_2 pressure. Several times during this process the residual mercury was allowed to run into the graduated part of the apparatus in order to expel any sea water that thus escaped the agitation, but the time required for this was counted out of the shaking. The apparatus was clamped upright, the lower cock opened in the usual manner, and the water allowed to run into the trap below. By carefully adjusting the screw, the water level was made to coin-

cide exactly with the upper entrance of the hole in the lower stop-cock stopper, and held there 1 minute in order to allow the apparatus to drain. The stop-cock was reversed and the mercury allowed to rise until it reached the same level as that in the leveling bulb that was raised to meet it. The mercury was held at this level 1 minute in order to allow the water trapped between the mercury and the glass to rise. The volume of water above the mercury was accurately observed and recorded, and the bulb raised so as to compress the gas by half the volume of this water. The lower stop-cock was now closed and the apparatus held (the fingers being at the extreme ends) on its side so that the sea water trapped below would not pass the Y-joint, and so that the gas and residual water above the mercury would pass into the wide part of the apparatus. It was agitated 1 minute in order to bring the residual water into equilibrium with the gas. The apparatus was clamped upright, care being taken to see that the mercury in the bulb was at the same level as that in the gas burette, and its level noted. The lower stop-cock was then opened. If the mercury in the burette changes more than 0.5 mm. the levels must be adjusted and the shaking repeated, but this seldom happens provided there is less than 0.1 cc. of water above the mercury. The mercury levels are now adjusted with the most extreme care, by means of the screw. In doing this for the first time, it is advisable to place two specks of dust on the mercury in the leveling bulb and sight over both of them simultaneously, in order to determine the horizontal. The leveling bulb is placed just behind the burette, and the screw adjusted so that the mercury meniscus in the burette and the two specks of dust in the bulb are brought into line. The volume of the gas is now accurately determined by reading the top of the water meniscus, and recorded. 0.5 cc. of half normal NaOH is placed in the cup and run into the burette. As the gas rises through the NaOH all of the CO_2 is absorbed, and no shaking is necessary. The mercury level is adjusted as before, but allowance must be made for the liquid over it. The length of this liquid is observed and one-tenth of it added to the top of the mercury meniscus, in order to determine an imaginary point through which to sight the two specks of dust on the mercury in the bulb. The volume of the residual gas is recorded and the barometer observed and corrected.

A large number of determinations have been made, many of them with carefully predetermined CO_2 contents; one of these determinations will show how the corrections were applied.

A solution having the same salt content as ocean water was prepared in a glass-stoppered volumetric flask from neutral salts, distilled water that had been boiled and cooled in a stream of CO_2-free air, and Na_2CO_3 that had been prepared from "reagent" $NaHCO_3$ by heating in a platinum dish for 30 minutes, cooling in a desiccator, and weighing with standard weights. 10 cc. of this artificial sea water were calculated to contain 0.687 cc. of CO_2.

The gas burette of the apparatus was calibrated with water while in the usual position, so that no further correction had to be applied to the actual volume of the gas. The remaining corrections were taken from the tables of Landolt-Börnstein-Roth. The temperature of the room was kept within 0.1° of 20°. The absorption coefficient of the sea water for CO_2 was taken as 0.777, from the work of Bohr.[1] The barometer was compared with a standard barometer, and corrected for gravity. Since there was 2 mm. capillary depression in the gas burette, this amount was subtracted from the corrected barometer reading, before making the calculations.

The volume of sea water above the mercury was read at 0.065 cc., but 0.014 (the combined volumes of the two menisci) must be added, making 0.079. The CO_2 as gas was equal to the total gas (0.778) minus the gas after absorption (0.195) or 0.583. The partial pressure of CO_2 on the water above the mercury was $0.583 \div 0.778 = 0.7494$ (of an atmosphere). The CO_2 absorbed by this water was $0.7494 \times 0.079 \times 0.777 = 0.04601$. Therefore the CO_2 pumped out of the water was $0.04601 + 0.583 = 0.62901$. Since this gas was expanded to 39 cc. when the mercury was lowered to the 50 cc. mark, the 11 cc. of water were under a partial pressure of $0.62901 \div 39 = 0.01613$, and contained $0.01613 \times 11 \times 0.777 = 0.1378$ cc. of CO_2. Therefore the total CO_2 was $0.62901 + 0.1378 = 0.76681$. The barometer (corrected) was 742.5 mm. and the log of the correction for temperature and pressure is about $-1+0.947196$. The log of the volume is about $-1+0.885$; therefore the total CO_2 reduced to

[1] Bohr, C., *Ann. Phys. u. Chem.*, 1899, lxviii, 500.

0° and 760 mm. is 0.6794 or 0.2 per cent too high. It should be noted that for great accuracy, the exact Cl content or salinity of the water should be determined and the absorption coefficient varied accordingly, but for most purposes it is sufficient to take the salinity of ocean water as about 35 per thousand, or Cl content as about 19 gm. per liter, in which latter case the absorption coefficient for CO_2 is 0.765 at 20°.

TABLE I.

Correction to Be Added to the Observed Volume of Water over the Mercury, on Account of the Meniscus Above and Below.

Length of tube containing 1 cc., *mm.*	50	60	70	80	90	100	110	120	130	140	150
Correction, *cmm.*	24	17	15	13.5	12.5	11.7	11.0	10.3	9.7	9.2	8.7

TABLE II.

Absorption Coefficient of Sea Water for CO_2 when the Temperature and Chlorine per Kilo of Sea Water Are Known.

18 gm. of Cl per kilo = 18.42 gm. per liter at 20° and 20 gm. per kilo = 20.5 gm. per liter at 20°. The absorption coefficient is given in cc. absorbed from one atmosphere of CO_2 by 1 liter of sea water.

Temperature, °C.	20	21	22	23	24	25	26	27	28	29	30
Cl = 18 per mill	772	750	730	718	688	667	652	638	624	610	596
Cl = 19 " "	767	745	725	714	684	663	648	634	620	606	592
Cl = 20 " "	761	740	720	710	680	659	644	630	616	602	588

Where sufficient skill is acquired to work rapidly, the second shaking and reading of the volume of water above the mercury may be omitted. A rubber tube of larger bore is used so that the mercury will rise so rapidly that little CO_2 is absorbed by the moisture on the walls. The first shaking is then the only shaking necessary and it may be done more rapidly with the apparatus in the vertical position. The absorption of CO_2 is delayed in hot weather by a film of vaseline from the stop-cock. It is better to grease the stop-cocks with a mixture of chicle, soft paraffin, and as little vaseline as possible.

THE STANDARDIZATION OF A NEW COLORIMETRIC METHOD FOR THE DETERMINATION OF THE HYDROGEN ION CONCENTRATION, CO_2 TENSION, AND CO_2 AND O_2 CONTENT OF SEA WATER, OF ANIMAL HEAT, AND OF CO_2 OF THE AIR, WITH A SUMMARY OF SIMILAR DATA ON BICARBONATE SOLUTIONS IN GENERAL.*

By J. F. McCLENDON.

(From the Physiological Laboratory of the University of Minnesota, Minneapolis.)

(Received for publication, March 14, 1917.)

The experiments on which this paper is based were all done by the author, but some of them have already been published (McClendon and Magoon, McClendon, 1916, *b*, and McClendon, Gault, and Mulholland). The main object of the present paper is the extension of this previous work by means of the new and slightly more reliable apparatus herein described. Sea water is much more difficult to investigate than blood, owing to its low buffer value and low CO_2 tension. It requires great care to measure 0.01 mm. in CO_2 tension, and yet this is about 4 per cent of the CO_2 tension of the sea. After the CO_2 tension is determined it may be changed by the solution of glass. The difficulties of making determinations with the hydrogen electrode are increased as the buffer value is lowered.

A Leeds and Northrup potentiometer and a 0.1 N KCl calomel electrode were used.[1] The mercury was redistilled in a Hulett still. The hydrogen electrode is shown in Fig. 1 as set up for

* Some of the apparatus was bought with a grant from the research fund of the Graduate School, and some was borrowed from the Marine Laboratory of the Carnegie Institution.

[1] Electrolytic calomel for this was kindly sent me by Professor G. A. Hulett.

the passage of the $H_2 + CO_2$ mixture through it. It was made of a tube of 24 mm. bore with a stop-cock at each end and a gold disk welded to a platinum wire fused through the glass. The gold disk was covered with palladium black by electrolyzing a 1 per cent filtered solution of palladium chloride, using a small platinum wire as anode. A 4 volt current was used, but this had to be cut down by reducing the size of the anode, especially at the beginning. If the palladiumization proceeds too fast, the palladium black does not stick to the cathode, but breaks off and moves to the anode in a cloud. After the electrode had been used a few times the black was dissolved off by concentrated nitric acid, and deposited anew.[2]

The gas mixture was admitted into the electrode by a swivel joint ground so true that it could be effectively closed with a water seal. The electrode was rotated 400 revolutions per minute by means of a Tiffany motor operating on a cork wheel or pulley. If the electrode contained only 2 cc. of sea water and its CO_2 tension at the start did not differ from that of the gas mixture more than 0.1 mm., equilibrium was reached by the passage of a liter of the gas mixture in the course of 30 to 40 minutes.

The gas mixer is also shown in Fig. 1 and is of 1 liter capacity, with the upper portion graduated and so narrow that 0.00001 liter can be read on it with ease. The 3-way stop-cock at the top allows the connecting tubes to be washed out with the gas to be introduced. Since the apparatus holds 25 pounds of mercury, the gas mixer and leveling bulb were wound with iron wire

[2] If the nitric acid is contaminated with chlorides or HCl, chlorine will be formed. If it is necessary to remove platinum black from electrodes, aqua regia is required and an abundance of chlorine is formed. The last trace of chlorine may be removed by electrolyzing a dilute solution of H_2SO_4, using the electrode as cathode. If, however, it is feared that a palladiumized electrode is contaminated with chlorine, it is safest to electrolyze distilled water with a higher voltage, as palladium black is attacked by mineral acids. The fact that palladium black is attacked by HCl may explain the rapid deterioration of palladiumized electrodes in gastric juice, which I have repeatedly observed. Another objection to palladium black is that it amalgamates instantaneously with mercury. The surface of hydrogen electrodes is "poisoned" by so many different substances that it often seems impossible to find the cause of the trouble. Both oxidizing and reducing gases may poison it.

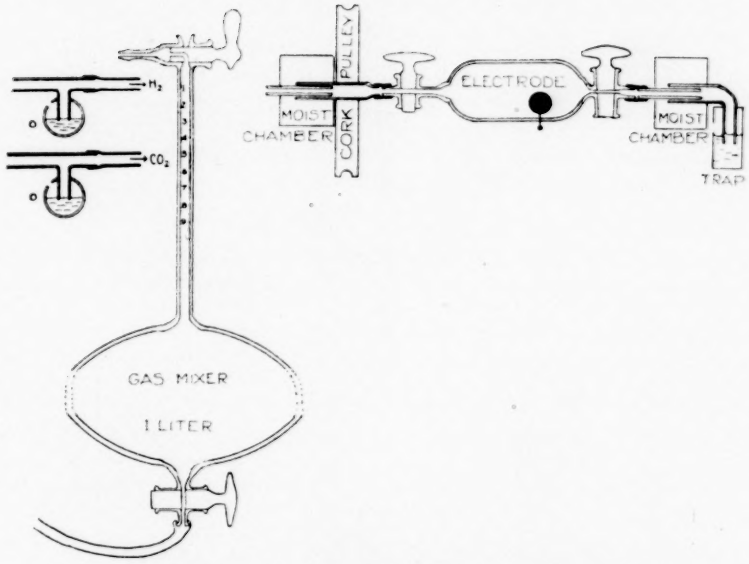

FIG. 1. Gas mixer and rotating electrode for determining the pH of a solution at any desired CO_2 tension. The gas mixer at the left is of 1 liter capacity down to the mark just above the lower stop-cock, and the numbers on the upper narrow portion mark 0.0001 liter. The mixer is filled with mercury and the CO_2 generator connected through the trap that delivers CO_2 at atmospheric pressure, to the upper stop-cock, which is first turned so that the gas escapes at the top and later reversed so as to admit the desired amount of gas. These operations are repeated with H_2 and the mixer is filled with it down to the 1 liter mark at the bottom. The remaining mercury is shaken with the gas to mix it. The mixer is now turned around and connected with the rotating electrode on the right through the swivel joint closed by a water seal in the moist chamber. A similar water seal connects the right hand end of the electrode with a trap to prevent the backward diffusion of air. The electrode (containing 1 or more cc. of the solution) is rotated 400 revolutions per minute by means of the cork pulley belted to a Tiffany motor while the gas mixture is slowly passed through it by displacement with mercury in the mixer. The stop-cocks at the two ends of the electrode are closed, and the one nearest the palladiumized disk (having been left ungreased) is immersed in the KCl bath and a wire hooked in the projecting loop from the palladiumized disk. The reading is now taken in the usual manner.

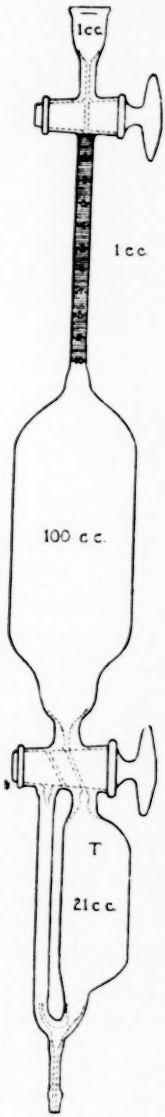

FIG. 2.

FIG. 2. Van Slyke apparatus slightly modified for determining the total CO_2 in sea water. A rubber suction tube, 1 mm. bore and 1 meter long, connects the lower end with a leveling bulb with cylindrical sides and filled with mercury. By raising the bulb the apparatus is filled with mercury. The upper stop-cock is closed and the bulb lowered so as to exhaust the apparatus. The bulb is raised without opening the upper stop-cock. The traces of air are now collected at the top and are forced out on opening the upper stop-cock. By means of careful manipulation of a pipette and the upper stop-cock, 10, 15, or 20 cc. of sea water are admitted, care being taken not to admit any air. It is permissible to leave one or two drops of this sea water in the 1 cc. cup at the top as this amount may be washed down by the introduction of 1 cc. of 2 N HCl in the same manner. A drop of mercury is placed in the cup to seal the stop-cock and the bulb lowered until the mercury falls to the etched mark near the bottom of the 100 cc. chamber. The lower stop-cock is closed and the apparatus is shaken laterally in the vertical position as vigorously as possible for 2 minutes (in case 20 cc. of sea water was used, 4 minutes). The sea water is now trapped off in the trap, T, and the mercury allowed to rise in the 100 cc. compartment until atmospheric pressure is attained in it and the lower stop-cock closed. At the end of 1 minute the amount of sea water above the mercury in the 1 cc. gas burette is measured, calculation being made for the two menisci. The apparatus is laid on its left side on a cushion and agitated 1 minute after the air is in the wide portion of the 100 cc. chamber. The apparatus is then clamped upright, the lower stop-cock opened, and the mercury levels are adjusted by means of a screw. The air volume in the 1 cc. burette is carefully measured. 1 cc. of 0.5 to 1.0 N NaOH is admitted from the cup into the gas burette to absorb the CO_2. In leveling the bulb to measure the gas residue, one-thirteenth of the height of the NaOH is reckoned as mercury. The total CO_2 that was in the original sea water is that absorbed plus that in the sea water above the mercury plus that in the sea water trapped in the trap, T. After these are calculated by means of the volumes, absorption coefficient, and CO_2 tension, they are added together and reduced to 0° and 760 mm. To do this it is necessary to know the barometric pressure and the capillary depression in the gas burette, which latter is subtracted from the former.

In the following example on 10 cc. of 0.576 N sea water at 20°, the absorption coefficient for CO_2 was 0.757, the barometric pressure 754 mm., and the capillary depression 2 mm. The CO_2 absorbed was 0.405 cc. and the gas volume before absorption 0.59 cc., making the CO_2 tension 0.687 (unity = barometric pressure). The sea water above the mercury was read as 0.075, to which was added the volume of menisci (0.0133) making 0.0883. The CO_2 in this sea water was $0.0883 \times 0.687 \times 0.757 = 0.0459$, which added to the absorbed CO_2 makes 0.4509. This occupied a space of 89 cc. over 11 cc. of acidulated sea water; hence there is in the trap $\frac{11}{89} \times 0.4509 \times 0.757 = 0.0422$, which, added to that already calculated, makes a total of 0.4931 cc., and which, reduced to 0° and 760 mm., is 0.442 per 10 cc., or 44.2 cc. per liter. Since the pH was 8.2 and the alkaline reserve 25, the total CO_2 agrees with Fig. 4.

that was run through boiling sealing wax as it was wound on. After the gases were measured into the mixer, they were thoroughly mixed by shaking up the little mercury remaining in it.

The hydrogen was generated from zinc and H_2SO_4 and passed through a large and a small wash bottle of $HgCl_2$ solution, and a small one of NaOH solution and one of H_2O. When the small wash bottle of $HgCl_2$ showed the first trace of discoloration with arsene, all the wash bottles were refilled. The CO_2 was generated from marble and HCl and washed with $NaHCO_3$ and H_2O. The gases were led to the gas mixer through traps (0, 0) that delivered them at atmospheric pressure, and a barometer in the same room was read during each experiment.

When a liter of the gas mixture had been passed through the revolving electrode, the stop-cocks were closed. The ungreased stop-cock was immersed in the usual KCl bath, a wire hooked into the platinum loop, and the electric potential determined. An identical gas mixture was made and passed through the electrode as before. If the potential remained the same, it was assumed that equilibrium with the gas mixture had been reached. In case it was desired to determine the total CO_2 on the same sample it was necessary to have 10 cc. of sea water in the electrode. The total CO_2 was determined by means of a modified Van Slyke apparatus for determining the CO_2 in serum (Fig. 2).

All the determinations were made in a constant temperature room automatically controlled within 0.2° by means of the apparatus previously described (McClendon, 1916, *a*). A damping vane in glycerol was added to the bimetallic thermoregulator. Some trouble was caused by arcing between the relay contacts carrying the 1,500 watt heating current. A lump of solder was placed on them so that it fell and rang a bell before a hot arc had time to form. Since a man's body heats 1 cubic meter of air about 0.5° per minute, rapid stirring of the air is needed. This was accomplished by means of a one-sixth horse power electric fan and two smaller fans. As the outdoor temperature varied from 0° to − 30°, no special arrangement for cooling the room was required, but the electric heat regulation was later found sufficient even with a window slightly raised and a vertical fan set in front of it.

Great care had to be taken in the manipulation of the Van Slyke apparatus and the reading of the menisci in order to reduce the error below 1 per cent. The greatest difficulty was experienced in greasing the stop-cocks so that they would hold a vacuum without soiling the interior of the apparatus with grease, but this difficulty disappeared on reducing the bore of the stop-cocks to 1 mm. A mixture of equal parts of soft paraffin, vaseline, and chicle, melted together and thoroughly stirred was found to be the best stop-cock grease. It was thinned with vaseline for lower temperatures. A source of error, the magnitude of which has not been determined, is the holding of some CO_2 by the thin film of sea water between the mercury and the glass, when the mercury is readmitted into the large compartment. This source of error is smaller in the modified apparatus. This error makes the values obtained too low, but in the standardization of the apparatus with Na_2CO_3 solution in CO_2-free distilled water, this error was so small as to be overcompensated by the absorption of CO_2 from the air by the solution in introducing it into the apparatus. In the case of sea water, some CO_2 is rarely lost and never gained.

The titration alkalinity or alkaline reserve was determined by titrating 100 cc. of sea water while boiling in an Erlenmeyer flask of resistance glass with 0.01 N HCl, using dibrom-*o*-cresolsulfophthalein as indicator. It usually required 1 cc. more than by the usual method of titration with phenolphthalein. The latter indicator was discarded since it was thought to be affected by some of the weak, non-volatile acids in sea water, although not by boric acid. The 0.01 N HCl is affected by the solution of glass about ten times as rapidly as 0.1 N acid (being noticeably changed in a month), and hence the stock solution was made of the latter and the former made from time to time by dilution. If a liberal supply of sea water is at hand, it is very desirable to make the titration on a liter of it with 0.1 N acid, as the end-point is not very sharp in any case. The water should remain yellow after vigorous boiling for 5 minutes after the last acid has been added.

In the experiments on which this paper is based, all liquid volumes were determined at 20° and the solutions used at 10°, 20°, and 30° without correction for volume change. The density

at 20° compared with distilled water at 4° is given. The concentration of the sea water is indicated by the normality of the chloride titration with silver nitrate and may be reduced to gm. per liter by multiplying by 35.46 and gm. per kilo by dividing the product by the density. The alkaline reserve is indicated by the number of cc. of 0.1 N HCl required to titrate a liter of the sea water. The total CO_2 is expressed as the number of cc. of dry CO_2 at 0° and 760 mm. that may be evolved from a liter of sea water by adding acid and boiling (but as stated above, the results are within the limits of accuracy of the micro method). The CO_2 tension is expressed as mm. of mercury, and may be reduced to atmospheres by dividing by 760. The pH was determined by the hydrogen electrode at the same temperature at which the CO_2 tension was regulated, but it was also found that the pH is not perceptibly affected by temperature provided there is no gain or loss of CO_2. The temperature change in hydrolysis of alkaline carbonates is compensated by the change in the dissociation of water.

Besides the Tortugas sea water previously investigated, determinations were made on 0.5366 N sea water from near San Diego[3] (alkaline reserve = 23.5, determined density = 1.0238), on 0.513 N sea water from Woods Hole, Massachusetts (alkaline reserve = 24, determined density = 1.0225), and especially on 0.576 N sea water (alkaline reserve = 25, determined density = 1.0254) taken from the Gulf Stream off Miami, Florida, and examined immediately on arrival by express. No difference was detected between these sea waters in the relation of CO_2 tension to pH. The relation of the total CO_2 to pH was affected only by the alkaline reserve (within the limit of error of the micro method).

It was found that the pH plotted against the logarithm of the CO_2 tension made a very gentle curve at higher CO_2 tensions. Within the limits given in Fig. 3 it was indistinguishable from a straight line (*i.e.*, the curvature is within the limits of error of the determinations). If the CO_2 tension remains constant the pH varies directly with temperature, 1° corresponding to 0.01

[3] This was sent by the Scripp Institution of Biological Research. The water from Woods Hole was sent by Professor A. W. Johnston, and that from Florida was taken by John Mills of the Carnegie Marine Laboratory and sent by Dr. Alfred G. Mayer.

pH. Fig. 3 may be used as a conversion table for finding the CO_2 tension of sea water from the pH and temperature. These values compare favorably with those of Henderson and Cohn in so far as comparison may be made.

When the pH is plotted against the total CO_2, the curvature is possibly greater, but the limits of error are greater, and the graph shown in Fig. 4 is as accurate as it has been possible to make from the data so far accumulated. This figure may be used as a conversion table for finding the total CO_2 from the pH and the alkaline reserve. The lines are not straight if extended. In one determination at pH 7.4 and alkaline reserve 25, the total CO_2 was 54 cc., whereas by the extended chart it would be 57 cc.

Since there is from twenty to thirty times as much CO_2 in the sea as in the air, the small surface of contact of these two cannot locally affect the CO_2 content of the sea water very much. The oxygen content of the sea water is more significantly affected, since it varies with the climatic zones, but the exchange of O_2 between sea and air is probably very slow. To the extent that the sea is a closed system, O_2 varies inversely to CO_2, due to the action of organisms, the possible error being 30 per cent. We may therefore use Fig. 4 for finding the oxygen content of sea water, provided the pH and alkaline reserve are known, and on the assumption that the respiratory quotient is unity and that a kilo calorie will raise the temperature of a liter of sea water 1°. It is obviously impossible to use these data in any attempt to determine the respiratory quotient of a marine animal in a sealed jar of sea water, but the data are valuable in indicating the limits of the oxygen supply, since the respiratory quotient of animals has been found to vary within narrow limits (0.7 to 1.0). The animal heat per cc. of CO_2 produced by the burning of carbohydrates is about 5 gm. calories, of proteins about 5.9, and of fats about 6.6. The error in estimating the total animal heat with the aid of Fig. 4 will be great only in case the respiratory quotient varies greatly from unity; *i.e.*, when a large proportion of fats and proteins is burned. It seems to be a fact that no gill-breathing animal has a temperature more than 2–3° above the surrounding water. After an inspection of Fig. 4 it seems incredible that even the recorded temperatures of aquatic animals could be maintained. The oxygen necessary for the generation

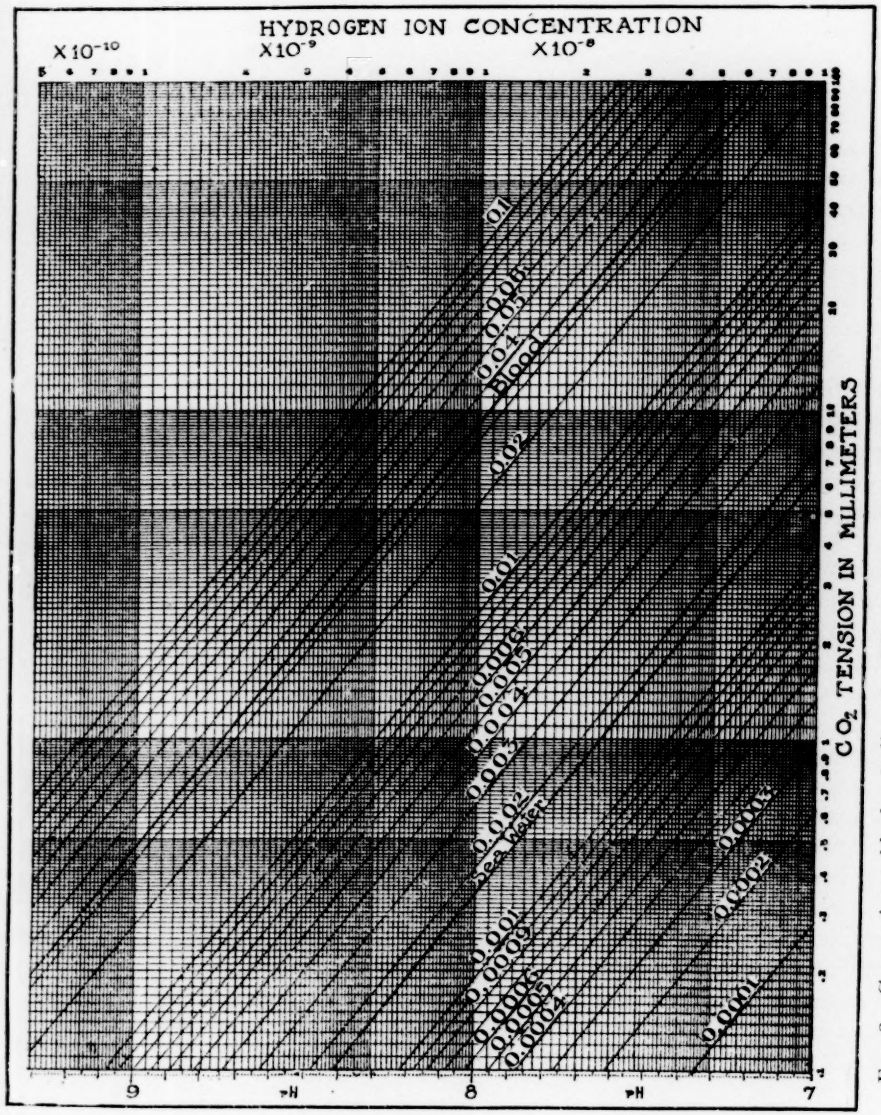

FIG. 3. Conversion table for finding the CO_2 tension at 20° of bicarbonate solutions, sea water, and normal human blood, from the pH. A simple calculation may be made for any other temperature since, if the CO_2 tension is kept constant, the pH varies directly with temperature, 1° corresponding to 0.01 pH. The CO_2 tension in mm. of mercury is marked on the ordinates and the hydrogen ion concentration is marked on the abscissæ. At the bottom is a scale for measuring the pH on the abscissa. The light diagonal lines denote pure bicarbonate solutions, the normality of which is indicated. The heavy diagonals denote blood and sea water

Fig. 4. Conversion table for finding the total CO_2 of sea water from the pH and alkaline reserve (cc. of 0.1 N HCl to titrate 1 liter of boiling sea water). The total CO_2 is measured on the ordinates and the pH on the abscissæ. Each diagonal is for sea water of the alkaline reserve indicated. At the left is given the expected O_2 per liter for tropical and again for temperate oceans. If the change in CO_2 is due to animal oxidations, the temperature change caused thereby is indicated in hundredths of a degree.

of heat must be carried from the gills to the interior by an aqueous fluid of high specific heat, and it would appear that the heat transfer must be nearly as effective as the oxygen transfer. The possession of hemoglobin, thus facilitating the oxygen transfer, should, however, allow a slightly higher body temperature to be maintained. A parallel case is indicated by Dallwig, Kolls, and Loevenhart in the comparison of the oxygen necessary to support a flame and maintain the temperature of a mammal. The flame can be maintained in 20 mm. O_2 tension made by rarefying the air, because the decreased oxygen content of the air is compensated by the decreased conduction of heat. But the flame is extinguished at 116 mm. O_2 tension made by diluting the air with nitrogen, because its specific heat is maintained. The mammal is unaffected by either process down to 40 mm. O_2 tension and perhaps farther, because the heat conductance depends largely on the specific heat of the blood, which is constant.

It follows from Figs. 3 and 4 that the total CO_2 plotted against the logarithm of the CO_2 tension forms a straight line. If Fox's data are plotted in this way, a straight line is approximated only for low CO_2 tensions. He used 500 cc. samples and hence his determinations were probably far more accurate than mine. He determined the CO_2 tension by titrating the CO_2 in air shaken with the sea water (and the sea water left in the bottle) by the Pettenkofer method. Perhaps the limits of error of this method could include the differences between Fox's data and mine. Or perhaps the air Fox analyzed had not come to equilibrium with the sea water. Fox discarded whole series of his data, and those remaining closely followed an empirical formula. I am not attempting to overthrow the determinations Fox made with his elaborate gas analysis apparatus by means of a micro method designed for other purposes than that for which it was used. I can only say that the two sets of data are for the present uncertain. It may be significant that his pH data are unreconcilable with mine. He determined these with the hydrogen electrode and also calculated them from the law of mass action and found the pH of sea water to be about 6, whereas I find it to be about 8. In this my results are in harmony with those of Palitzsch, Henderson and Cohn, and others.

Since Figs. 3 and 4 may be used as conversion tables provided the two titrations are made and the temperature and pH are determined, it seems desirable that a method be perfected for determining the pH on shipboard or in poorly equipped laboratories. The colorimetric method is the only one adaptable, and phenolphthalein is objectionable because errors in concentration simulate changes in pH. The buffer mixtures in use have a very different salt concentration from sea water, and the salt error has to be determined for every new indicator. For these reasons it seemed desirable to use the new indicators recommended by Lubs and Clark with buffer mixtures having the same salt concentration as sea water. Sea water varies in concentration, but the range from 0.4 to 0.6 N is perhaps great enough for all purposes. It was also desirable to increase the concentration of the buffers so that they will be less affected by the solution of glass. Since the solubility of buffers is reduced by the increase in salt concentration, the latter was made to correspond to 0.4 N sea water and a slight correction applied for 0.5 N sea water and a greater one for 0.6 N sea water. In actual practice the correction for 0.5 N sea water has already been made on the labels on the sealed tubes of buffers. A correction of 0.05 pH is then indicated for 0.6 N sea water and a similar correction but in the reverse direction for 0.4 N sea water. The salt error between the buffer mixtures and 0.5 N sea water was redetermined with the hydrogen electrode a large number of times at different temperatures and for both thymolsulfophthalein and *o*-cresolsulfophthalein, and likewise with 0.6 N sea water, but I do not believe this absolutely necessary. A moderate dilution of sea water with distilled water does not appreciably change the pH if the CO_2 tension is near that of the atmosphere and it is not agitated with or exposed to the air. It is then only necessary to dilute the sea water to determine the salt error with any new indicator, taking the 0.4 N sea water as the standard for the calculation of the correction in the pH of 0.5 and 0.6 N sea water. The salt error for the above indicators holds for phenolsulfophthalein and probably for all sulfophthalein indicators.

The buffer mixtures are made from two stock solutions "boric" and "borax," kept in Squibb's automatic burettes provided with soda-lime tubes. The distilled water used in making them is

boiled 15 minutes to rid it of CO_2 and cooled in a tightly stoppered, narrow necked flask or by means of a stream of CO_2-free air. The boric acid is recrystallized and dried in a desiccator (not by heat). The borax is recrystallized and dried in dry air short of efflorescence. The NaCl should be pure, but drying by heat is unnecessary because the error due to occluded moisture in the crystals is too small to cause a noticeable difference in the color of the indicators.

The "boric" contains 18.6 gm. of boric acid and 22.5 gm. of NaCl to the liter.

The "borax" contains 28.67 gm. of borax and 19 gm. of NaCl to the liter, and has the same salt action as the "boric" on the indicators. If kept at a low temperature, borax crystallizes out and must be made uniform in solution before mixtures are made. The same applies to the mixtures. The desired indicator is added to the stock solutions to the extent of 10 mg. to the liter, or added to the mixtures in the same proportion. In order to avoid dilution of the buffers and also to avoid the necessity of weighing the indicator for each solution, it was made up in 0.1 per cent solution in alcohol redistilled over sodium. Thymolsulfophthalein requires the addition of a little NaOH to get it into solution at this concentration and hence the solution becomes less sensitive if allowed to absorb CO_2. If 0.01 per cent aqueous solution of the indicator is used for addition to sea water, the concentration of the sea water after the addition is used in calculating the salt error, since this dilution of 10 per cent corresponds to 0.025 pH in the salt error.

30 cc. of each mixture containing the indicator were sealed up (by fusing the glass) in a "Nonsol" test-tube of exactly 24 mm. bore without introducing CO_2 from the blast lamp. Since these indicators are impure, it is necessary to test each lot, both as to concentration and range. The concentration may be tested by comparison with a sealed tube of the original indicator in distilled water, the concentration being the same as in the mixtures. In order to test the range, it is necessary to save samples of the stock solutions without indicator. This may be done by making mixtures, one near the middle or one near each end of the range of the indicator and sealing them in "Nonsol" tubes. It is then only necessary to cut off the tip of the seal and introduce the

new sample of indicator in order to test it. The "boric" allows the growth of mold, but the "borax" is antiseptic. The tubes containing a large proportion of "boric" should be sterilized by immersion in water up to the air space and boiling the water.

To facilitate comparison of the tubes, a colorimeter was made by placing stereoscope prisms together in a sharp line and placing two of the tubes at such a distance behind them that the centers of the images were brought together in a sharp line. A thin milk-white (opal) glass was placed immediately behind the tubes to disperse the direct sunlight or mazda light passed through "daylite" glass.[4]

The mixtures are given in the table on the following page.

The salt action on the indicators is approximately directly proportional to the salt concentration over the range from 0.1 to 0.6 N, the difference in salt error of two sea waters being one-half to three-fifths the difference in normality, and increase in salinity causing the same color change as increase in alkalinity (increase in pH).

The useful range of *o*-cresolsulfophthalein is from the first of the series to pH 8.3 and of thymolsulfophthalein from 7.9 to the last of the series. For the surface water of the open sea one indicator is about as good as the other, except in the tropics, where thymolsulfophthalein is the best. In the study of the respiration of marine animals *o*-cresolsulfophthalein or α-naphtholsulfophthalein should be used.

A more or less definite relation between the pH and the solubility of calcium salts in the sea water seems to exist. Dittmar showed that there is less calcium in the surface waters than in the deep waters of the ocean, and Sörensen and Palitzsch showed that the pH is higher in the surface waters. It is difficult to study this question *in vitro*, owing to the relative stability of the supersaturated solutions of $CaCO_3$, and the existence of it in solid form as aragonite, calcite, lublinite, and vaterite, with different solubilities. Presumably the surface water over lime mud flats in the tropics is saturated with calcite, or nearly so. If $CaCl_2$ is added to this water, in the form of a concentrated solution, no precipitate occurs, but if the pH is only slightly increased, $CaCO_3$ begins to deposit on the glass, and it takes relatively little increase in the pH to cause a precipitate

[4] The sealed tubes and colorimeter may be obtained from Hynson, Westcott and Dunning, Baltimore. The tubes are labeled for 0.5 N sea water.

"Boric."	"Borax."	pH of sea water.			"Boric."	"Borax."
		0.4 N; all indicators.	0.5 N; sulfophthalein.	0.6 N; sulfophthalein.		
per cent	*per cent*				*cc.*	*cc.*
79.5	20.5	7.50	7.45	7.40	23.90	6.1
78	22	7.55	7.50	7.45	23.4	6.6
76	24	7.60	7.55	7.50	22.8	7.2
74	26	7.65	7.60	7.55	22.2	7.8
72	28	7.70	7.65	7.60	21.6	8.4
70	30	7.75	7.70	7.65	21.0	9.0
68	32	7.80	7.75	7.70	20.4	9.6
66	34	7.85	7.80	7.75	19.8	10.2
64	36	7.90	7.85	7.80	19.2	10.8
62	38	7.95	7.90	7.85	18.6	11.4
60	40	8.00	7.95	7.90	18.0	12.0
58	42	8.05	8.00	7.95	17.4	12.6
56	44	8.10	8.05	8.00	16.8	13.2
54	46	8.15	8.10	8.05	16.2	13.8
52	48	8.20	8.15	8.10	15.6	14.4
51	49	8.22	8.17	8.12	15.3	14.7
49.5	50.5	8.25	8.20	8.15	14.85	15.15
47	53	8.30	8.25	8.20	14.10	15.9
44.5	55.5	8.35	8.30	8.25	13.45	17.55
42	58	8.40	8.35	8.30	12.60	17.4
39.5	60.5	8.45	8.40	8.35	11.85	18.15
37	63	8.50	8.45	8.40	11.10	18.9
34.5	65.5	8.55	8.50	8.45	10.35	19.65
32	68	8.60	8.55	8.50	9.6	20.4
29	71	8.65	8.60	8.55	8.7	21.3
26	74	8.70	8.65	8.60	7.8	22.2
23	77	8.75	8.70	8.65	6.9	23.1
20	80	8.80	8.75	8.70	6.0	24.0
17	83	8.85	8.80	8.75	5.1	24.9
14	86	8.90	8.85	8.80	4.2	25.8
11	89	8.95	8.90	8.85	3.3	26.7
8	92	9.00	8.95	8.90	2.4	27.6
4.5	95.5	9.05	9.00	8.95	1.35	28.65
1	99	9.10	9.05	9.00	3.0	29.7

throughout the solution. The size of grain makes a difference in the solubility of the precipitate, but if time for equilibrium is allowed, the small grains will change into less soluble crystals. According to Irvine and Young, sea water will dissolve 125 parts per million of crystallized $CaCO_3$. Rona and Takahashi determined the total Ca and the pH in mixtures of Na and Ca carbonates, bicarbonates, and chlorides, in contact with pre-

cipitated $CaCO_3$. The concentration of the chlorides varied, but the alkaline reserve was about 160 in each. The following table gives the pH and the normality of $\frac{1}{2}$ Ca.

pH	6.65	6.80	6.89	6.97	7.03
$\frac{1}{2}$ Ca N	0.00985	0.0064	0.00492	0.00455	0.00405

In sea water the pH is about 8, the alkaline reserve is about 25, and the normality of $\frac{1}{2}$ Ca about 0.1 The greater solubility of Ca in sea water notwithstanding the greater pH is due to decreased concentration of total CO_2 and possibly to the increased concentration of chlorides. Since the chlorides are nearly constant and the alkaline reserve, total CO_2, and Ca are interdependent, any change in the pH must cause a change in the solubility of Ca. When the pH is sufficiently raised, $CaCO_3$ is precipitated and the Ca content and alkaline reserve are lowered. At present the law of mass action cannot be applied to such complex mixtures containing divalent salts whose second dissociation constant is unknown. Harkins and Pearce found that the addition of a salt with a common polyvalent ion may increase rather than decrease the solubility of a polyvalent salt. It is possible that the presence of $CaCl_2$ may increase rather than decrease the solubility of $CaCO_3$ in sea water. The solubility product law as applied to univalent salts does not apply without modification to polyvalent salts of certain types.

In a previous paper (McClendon, 1916, *b*) I described an experiment in which the CO_2 tension of the air was determined by drawing it through sea water and then determining the pH of the sea water. A considerable time was required to reach equilibrium, and therefore special precautions had to be taken to prevent contamination or evaporation of the sea water. In order to avoid these precautions, I have experimented with bicarbonate solutions of such low alkaline reserve that equilibrium is reached quickly. In the meantime Higgins and Marriott published similar experiments, but their method was not sensitive enough for my purposes. I found that the pH of a 0.0003 N $NaHCO_3$ solution as measured colorimetrically with phenolsulfophthalein and Sörensen's phosphate mixtures changed with low CO_2 tensions as shown in Fig. 3, for 20°. The pH varied directly with the temperature, 1° corresponding to 0.01 pH. Hence the 0.0003 N line in Fig. 3 may be used as a conversion table to find the CO_2 tension of the air from the pH and temperature. If the temperature is 19°, 0.01 is added to the pH before using the conversion table, and if the temperature is 27°, 0.07 is subtracted from the pH before using the conversion table.

The phenolsulfophthalein was made up in 0.04 per cent solution in absolute alcohol and 5 cc. of it were added to 300 cc. of the phosphate solutions. The latter, M/15 solutions of KH_2PO_4 and Na_2HPO_4, were mixed in the following proportions:

pH	Na_2HPO_4	KH_2PO_4	pH	Na_2HPO_4	KH_2PO_4
7.00	18.2	11.8	7.40	24.2	5.8
7.05	19.0	11.0	7.45	24.7	5.3
7.10	19.8	10.2	7.50	25.2	4.8
7.15	20.6	9.4	7.55	25.6	4.4
7.20	21.4	8.6	7.60	26.0	4.0
7.25	22.2	7.8	7.65	26.4	3.6
7.30	23.0	7.0	7.70	26.7	3.3
7.35	23.6	6.4			

These mixtures were sealed in "Nonsol" test-tubes of exactly 24 mm. bore. The apparatus for testing the air was shaped similar to a Dutchman's pipe. The bowl consisted of a short piece of "Nonsol" test-tube of exactly 24 mm. bore, and the stem of a piece of "Nonsol" tubing of about 7 mm. bore and a meter long. About 10 cc. of 0.0003 N $NaHCO_3$ solution, containing the same concentration of phenolsulfophthalein as the phosphate mixtures contain, was placed in the bowl of the pipe and suction applied to the mouthpiece by means of the mouth of the operator, so that the air passed up through the solution in bubbles in the pipe-stem. This process was continued until no further color change occurred in the solution, which was then allowed to run back into the bowl and its color compared with that of the sealed tubes. A thermometer was then placed in the bowl and the temperature of the solution determined.

By making the bowl of the pipe 10 mm. bore, it was found possible to use Hynson and Westcott phenolsulfophthalein tubes made for determination of pH of blood dialysate, but with less accuracy.

The main difficulty experienced has been in keeping a 0.0003 N solution of $NaHCO_3$. A gold flask would be too expensive and a pure silver flask hard to procure. It seems unwise to invest in a gold-plated flask without the guarantee that the plate will remain perfect. Ceresin bottles may be used, but ceresin and

rubber lacquer peeled off. I silvered the interior of a flask of resistance glass. The silvering peeled off in the upper part of the neck, and was affected by sulfur from the rubber stopper. If gold is burned on the glass in a layer thick enough to be opaque, it peels off. If the layer is thinner, it may stick for a long time. I gilded one resistance flask as follows: Campana's bright liquid gold was thinned with banana oil and poured into the flask and poured out, and dried thoroughly in an inverted position by means of a current of air. It was then heated in the blast lamp. Perhaps better results might be obtained by substituting regular thinner made for china painter's gold, and a china kiln. Irving Langmuir has described[5] a process of gilding by volatilizing gold on an electrically heated tungsten filament in high vacua. Ceresin bottles do not absorb the indicator and are perhaps the best for holding the bicarbonate solution colored with the indicator. The indicator undergoes some change in absolute alcohol.

The time required for reaching equilibrium is lessened if air is passed through the stock solution of bicarbonate so as to remove some of the CO_2. The stock solution may be made by passing CO_2 or the breath through a NaOH or Na_2CO_3 solution. The more CO_2 the solution contains, the less it attacks glass, silica, and other flask materials, and rubber stoppers. Sulfur-free rubber stoppers are very desirable for closing the flasks. On a trip from San Francisco to Samoa and return, Dr. A. G. Mayer found the CO_2 tension of air over the Pacific to vary from about 0.23 to 0.3 mm. by this method, whereas the variation of the CO_2 tension of the sea surface was much greater and not correlated with that of the air. This indicates that diffusion of CO_2 between air and sea is very slow.

When the CO_2 tension is known, the per cent of CO_2 in the air may be found by multiplying by 100 and dividing by the barometric pressure in mm.

In the determination of the CO_2 of the air and in other studies, a number of $NaHCO_3$ solutions were investigated, as shown in Fig. 3. These were preliminary determinations, and no special accuracy is claimed for them. The chief source of error probably lies in failure to reach equilibrium, as shown by the follow-

[5] Paper presented before the American Physical Society, 1916.

ing example on sea water. A rapid stream of CO_2-free hydrogen was passed through a few cc. of sea water and at the end of 270 minutes the alkali had been reduced to carbonate, but only a small fraction to NaOH, and yet CO_2 was being continuously eliminated at the end of the experiment. The experiments were accurate enough, however, to indicate, at least within certain limits, that at constant temperature the pH of these solutions is proportional to the logarithm of the alkaline reserve and inversely proportional to the logarithm of the CO_2 tension. If the CO_2 tension remains constant, a rise of 1° in temperature causes an increase of 0.01 pH, whereas if the total CO_2 content remains constant, the pH is not affected by temperature. At constant CO_2 tension and temperature, as shown by Fox, the total CO_2 is directly proportional to the alkaline reserve within a slight error equal to the CO_2 absorbed by distilled water under the same conditions.

My experiments indicate, at least within certain limits, that the slope of the curves, as in Fig. 3, is the same for sea water, blood, and other biological media (except those exceptionally rich in phosphates) as it is for bicarbonate solutions. Neutral salts slightly decrease the hydrolysis of bicarbonate and decrease the pH. The chief buffer in these media is bicarbonate, and other buffers are not present, even in blood, in sufficient concentration to change the slope of the curve greatly, at least in its upper regions, but the buffer action of proteins in blood comes into play when the blood is made strongly alkaline by the elimination of CO_2, the curve for blood in Fig. 3 being slightly incorrect in the lower CO_2 tensions.

It would be of interest in this connection to know the concentration of bicarbonate in normal blood, but the presence of phosphates and organic matter makes titration very uncertain. (The compensation dialysis method might yield somewhat more accurate results.) The bicarbonate concentration of different bloods is practically proportional to the total CO_2 content at the same CO_2 tension, and hence Van Slyke's method of determining the alkaline reserve of plasma might be used to determine the bicarbonate, provided the conversion factors from his units to the titration units were known. The bicarbonate content of blood does not often exceed 0.04 N, as will be shown in a later paper.

Since the concentration of neutral salts is only about a fourth as great as in sea water, their effect in reducing the hydrolysis is slight. The bicarbonate concentration is probably but little more than that of a pure bicarbonate solution that has the same pH at the same CO_2 tension and temperature, and hence could be estimated by means of a chart constructed on the same principles as Fig. 3 but somewhat more accurate in this region.

The above views seem to differ somewhat from those of Henderson and Cohn on sea water. Henderson and Cohn found it necessary to add 0.0015 M H_3BO_3 to a liter of alkaline NaCl solution in order to make it behave like sea water in regard to pH. My experiments were at variance to this, but since CO_2 tension is one of the most difficult factors to determine exactly, other methods seemed necessary to determine the concentration of non-volatile buffers in sea water. A serviceable method was found to be the titration of CO_2-free sea water with CO_2-free NaOH in the hydrogen electrode. It is difficult to maintain the sea water and NaOH absolutely CO_2-free, and the first trace of CO_2 is immediately titrated as non-volatile buffer. By titrating directly into the electrode shown in Fig. 5 (after removal of the trap at the top) the results could be closely duplicated, and are shown in Fig. 6. The titration must be done rapidly and not carried beyond pH = 10 owing to the precipitation of (carbonates if present) phosphates (borates?) and finally hydroxides of Ca and Mg. Sea water has hardly more non-volatile buffer than artificial sea water previously described (McClendon, 1916, *b*). The concentration of non-volatile buffer in Atlantic, Pacific, and Gulf Stream water is practically identical. On the contrary, the non-volatile buffer in the solution used by Henderson and Cohn is very much higher in concentration. Boric acid was detected in all samples of sea water, but it is evidently in less concentration than 0.0015 M. The phosphoric acid quantitatively recovered from sea water is negligible.

In attempting to confirm these findings by plotting the pH against the CO_2 tension, as was done by Henderson and Cohn, the first experiments were apparently vitiated by the presence of some air in the CO_2. At any rate very irregular results were obtained. In an attempt to clear up the doubt aroused by these results, a large number of determinations were made on a varied

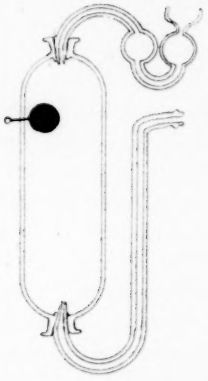

FIG. 5. Hydrogen electrode for CO_2-free titrations, for standardizing buffer mixtures, and determining the salt action on indicators (for which purpose its diameter is made the same as the colorimetric tubes). The tube admitting hydrogen at the bottom is ground to fit the openings at each end of the electrode, so that the latter may be inverted when it is desired to change the height of the palladiumized disk. In making the electrometric titration the trap at the top is discarded, and the burette tip inserted in its place. Glass wool moistened with distilled water and wrapped around the burette tip serves as a trap to keep out O_2 from the air above. The lower ground joint is ungreased because it is immersed in the KCl bath for electrolytic connection with the calomel electrode.

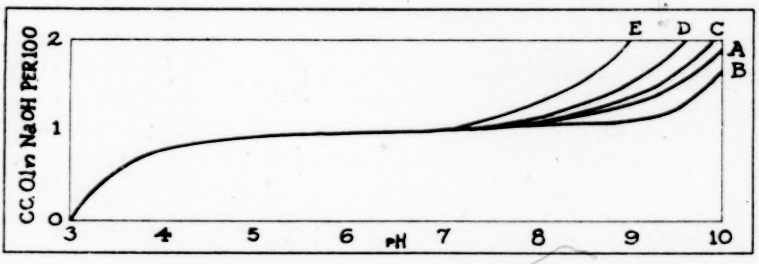

FIG. 6. Electrometric titration of CO_2-free sea water and artificial sea waters. The pH is measured on the abscissæ and the number of cc. of CO_2-free NaOH on the ordinates. The left hand ends of all the curves practically coincide, the right hand ends are marked as follows: A = sea water. B = artificial sea water of formula given by McClendon (1916, *b*). C = the same + 0.00017 M H_3PO_4. D = the same + 0.001 M H_3BO_3. E = the same + 0.0015 M H_3BO_3.

series of solutions of alkaline reserve = 25, given in the following list:

A. 0.0025 N $NaHCO_3$.
B. 0.0025 " " + 0.025 N NaCl.
C. 0.0025 " " + 0.0025 " $CaCl_2$.
D. 0.0025 " " + 0.025 " "
E. 0.0025 " " + 0.025 " $MgCl_2$.
F. Artificial sea water, alkaline reserve = 25 (for formula see McClendon, 1916, *b*).
G. Artificial sea water + 0.0015 M H_3BO_3.
H. " " " + 0.001 " "
I. " " " + 0.0008 " "
J. " " " + 0.00017 " H_3PO_4.

Some of these experiments indicate that $\frac{1}{2}$ $Ca^{..}$ reduces the hydrolysis of the bicarbonate more than $Na^{.}$ does, but this note is made merely as a suggestion for further research. The experiments in general indicate that small amounts of neutral salts or non-volatile buffers have little effect on the pH at constant CO_2 tension. Some deviations from this rule were ascribed to impurities in the salts. The $CaCl_2$ used in the last experiments was dissolved in absolute alcohol that had been redistilled over sodium, evaporated, fused in a platinum dish, dissolved in distilled water, and carefully neutralized.

Henderson and Cohn, using the indicator method of Palitzsch, record an effect of salinity on the pH of sea water at constant CO_2 tension. It is not clear whether they mean a simple change in the concentration of neutral salts or whether the alkaline reserve was also changed. If the neutral salts alone were changed, the change in pH was in the wrong direction for the effect of salts on the hydrolysis of bicarbonate, but was in the right direction for the salt effect on phthalein indicators. According to my experiments, neither the salinity nor the alkaline reserve in sea water of the tropical or temperate oceans change sufficiently to change noticeably the relation of pH to CO_2 tension, although the alkaline reserve does change sufficiently to affect the total CO_2 greatly.

BIBLIOGRAPHY.

Bohr, C., *Ann. Phys. u. Chem.*, 1899, lxviii, 500.
Clark, W. M., and Lubs, H. A., *J. Biol. Chem.*, 1916, xxv, 479.
Dallwig, H. C., Kolls, A. C., and Loevenhart, A. S., *J. Biol. Chem.*, 1915, xx, p. xxxii.
Dole, R. B., *Carnegie Institution of Washington, Publication 182*, 1914, 69.
Fox, C. J. J., *Tr. Faraday Soc.*, 1909, v, 68; Conseil perm. int. pour l'explor. de la mer, Copenhagen, 1909.
Frary, F. C., and Nietz, A. H., *J. Am. Chem. Soc.*, 1915, xxxvii, 2263, 2268.
Haas, A. R., *Science*, 1916, xliv, 105.
Harkins, W. D., and Pearce, W. T., *J. Am. Chem. Soc.*, 1916, xxxviii, 2679.
Hasselbalch, K. A., *Biochem. Z.*, 1913, xlix, 451.
Henderson, L. J., *Am. J. Physiol.*, 1908, xxi, 173, 120, 427.
Henderson, *Ergebn. Physiol.*, 1909, viii, 254; *J. Biol. Chem.*, 1911, ix, 403.
Henderson, L. J., and Cohn, E. J., *Proc. Nat. Acad. Sc.*, 1916, ii, 618.
Higgins, H. I., and Marriott, W. M., *J. Am. Chem. Soc.*, 1917, xxxix, 68.
Krogh, A., *Meddelelser om Grönland*, 1904, xxvi, 333, 409.
Lubs, H. A., and Acree, S. F., *J. Am. Chem. Soc.*, 1916, xxxviii, 2772.
Lubs and Clark, *J. Wash. Acad. Sc.*, 1915, v, 609; 1916, vi, 483.
McClendon, J. F., *J. Biol. Chem.*, 1916 (*a*), xxiv, 519.
McClendon, *J. Biol. Chem.*, 1916–17 (*b*), xxviii, 135.
McClendon, J. F., Gault, C. C., and Mulholland, S., *Carnegie Institution of Washington, Publication 251*, 1917, 21.
McClendon, J. F., and Magoon, C. A., *J. Biol. Chem.*, 1916, xxv, 669.
Marriott, W. M., *J. Am. Med. Assn.*, 1916, lxvi, 1594.
Palitzsch, S., *Biochem. Z.*, 1911, xxxvii, 116.
Rona, P., and Takahashi, D., *Biochem. Z.*, 1913, xlix, 370.
Sörensen, S. P. L., *Ergebn. Physiol.*, 1912, xii, 393.
Veatch, J. A., *Proc. Cal. Acad. Sc.*, 1859, ii, 7.

STUDIES OF ACIDOSIS.

I. THE BICARBONATE CONCENTRATION OF THE BLOOD PLASMA; ITS SIGNIFICANCE, AND ITS DETERMINATION AS A MEASURE OF ACIDOSIS.*

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I. Blood Bicarbonate and Acidosis.

Free carbonic acid is present in the body fluids in such concentration that it automatically converts into bicarbonate all bases not bound by other acids. *The bicarbonate therefore represents the excess of base* which is left after all the non-volatile acids have been neutralized and is available for the immediate neutralization of further acids. In this sense it constitutes the *alkaline reserve of the body.* The bicarbonate concentration of the blood is representative of that of the body fluids in general, and is normally maintained at a definite level. Entrance of free acids reduces it to an extent proportional to the amount of the invading acid.

While in data to be published in this and subsequent papers we believe that we have broadened the foundation of facts on which the above statements stand, the latter are either contained in the propositions laid down by Henderson (1908, *b*; 1909, *b*), as the result of observations by himself and others,[1] or are self-evident corollaries of those propositions. They establish the blood bicarbonate as a criterion of the acid-base balance of the body. Accordingly, for use in the present series of papers, we define *acidosis* as *a condition in which the concentration of bicarbonate in the blood is reduced below the normal level.* The definition appears a necessary preliminary because of present confusion in the literature, different authors regarding acidosis differently as "acid intoxication," as a condition in which acetone bodies are formed, or as an actual increase in the hydrogen ion concentration of the blood.

Acidosis in the sense defined may result, as in diabetes, from such an overwhelmingly rapid production of acids that even an apparently undamaged eliminating mechanism working at several times the usual rate cannot dispose of them. Or it may result, as in nephritis, from inability to eliminate acids even at the moderate rate at which normal metabolism produces them. In either case the retained acid decomposes body bicarbonate, forming in its place the salt of the invading acid.

[1] Henderson's monograph and the papers by Henderson and Palmer contain so complete an exposition of the mechanism by which phosphates and the kidneys assist in maintaining body neutrality that this portion of the subject is given minimum consideration in the present paper.

The bicarbonate not only represents the alkaline reserve of the body, but its normal concentration in the blood is so definite that it constitutes a physiological constant. The blood plasma of the normal adult contains 50 to 65 per cent of its volume of CO_2 gas bound as bicarbonate. The limits of variation are similar in magnitude to those of the pulse rate. By utilizing as a standard the normal bicarbonate concentration we can reduce the term "acidosis" to as definite a meaning as "fever" or "tachycardia." In each case a condition is indicated in which one of the physiological constants falls or rises to an abnormal level. The possible causes are numerous, but the result, in the case of acidosis a lowering of the blood bicarbonate, is an accurately definable and determinable phenomenon. Like accelerated pulse rate or increased temperature, it may occur temporarily even in health, *e.g.*, as the result of muscular exertion and the consequent lactic acid formation (Christiansen, Douglas, and Haldane, 1914). It is not necessarily a pathological condition in itself, but is a symptom of disturbed function. Like fever or tachycardia, however, acidosis in itself becomes a danger when it has reached a sufficient degree of intensity.

The hydrogen ion concentration, C_H, of the blood is a physiological constant even less variable than the plasma bicarbonate (Lundsgaard, 1912), the normal value of approximately $10^{-7.45}$ being maintained with the utmost tenacity by the normal organism. Nevertheless, as a standard for measuring changes in the acid-base balance, it appears less desirable than the bicarbonate, for the reason that, while the bicarbonate decreases progressively as soon as the normal excess of bases over acids begins to be depleted, rise of the blood C_H is usually one of the latest changes that follow.

Benedict (1906) and Michaelis (1914, p. 105), for example, have observed in diabetic acidosis an increased C_H only after terminal coma had set in, and Peabody (1914) has made similar observations in the acidosis of cardiorenal disease. In both types of cases coma occurs only after the blood bicarbonate has been reduced to a fraction of its normal value.

The reason for the lateness of the stage at which increase in blood C_H appears is the fact that, until a large part of the bicarbonate has been exhausted, the organism can, by accelerated

respiration, maintain the ratio[2] $\frac{H_2CO_3}{NaHCO_3}$ in the arterial blood at its normal value. And the C_H, being directly proportional to this ratio, is thereby also kept normal.

The manner in which the body uses carbonic acid and bicarbonate in order to maintain its neutrality has been most clearly described by Henderson in his monograph (1909, *b*). The normal C_H is maintained by the mechanism for keeping the $\frac{H_2CO_3}{NaHCO_3}$ constant. From the law of mass action

$$C_H = K \frac{H_2CO_3}{CO'_3} = K \frac{H_2CO_3}{\lambda NaHCO_3}$$

λ being the degree of dissociation of $NaHCO_3$ into Na^+ and HCO'_3 in the blood, and K the ionization constant of H_2CO_3. Since λ varies but slightly within the range of conditions encountered within the blood plasma, one may state that in the plasma *the hydrogen ion concentration varies directly as the value of the molecular ratio* $\frac{H_2CO_3}{NaHCO_3}$. Hasselbalch (1916, *b*) has shown that this law holds so accurately that he regards the determination of the $\frac{H_2CO_3}{NaHCO_3}$ ratio as an even more reliable means than the gas chain for determining blood hydrogen ion concentration. Whenever, either by increased rate of CO_2 production or by decomposition of $NaHCO_3$ by acid, the ratio $\frac{H_2CO_3}{NaHCO_3}$ is increased, the C_H of the blood is proportionately increased, and stimulates respiration. More rapid ventilation follows until the H_2CO_3 of the blood is so reduced that the normal $\frac{H_2CO_3}{NaHCO_3}$ ratio, and consequently the normal C_H, is restored.

The respiratory response is so sensitive to this stimulus that Campbell, Douglas, Haldane, and Hobson (1913) observed that an increase of only 1 mm. in the CO_2 tension accelerated the rate of ventilation 60 per cent, and Boothby (1915) has observed that the heart output is similarly increased in the effort to rid the body of excess CO_2.

We find that plasma, obtained by drawing blood from the arm vein and centrifuging at once, contains at 37° and normal CO_2

[2] A minor portion of the combined carbonic acid of the plasma is, of course, neutralized by bases other than sodium; but as the acid-neutralizing power of the strong bases does not differ greatly we follow Henderson's convenient practice of using "$NaHCO_3$" to indicate the total bicarbonate.

tension approximately 60 volume per cent of CO_2 gas bound as bicarbonate. The concentration of CO_2 in the form of H_2CO_3 calculated from the average arterial CO_2 tension of 42 mm. is 3 volume per cent $\left(\frac{42}{760} \times 100 \times 0.54 = 3.0\right.$, 0.54 being the solubility coefficient of CO_2 in blood plasma at body temperature, as determined by Bohr). Consequently the normal ratio

$$\frac{H_2CO_3}{NaHCO_3} = \frac{3}{60} = \frac{1}{20}$$

a value which agrees approximately with that calculated from the known values of the constants in the equation[3]

$$\frac{H_2CO_3}{NaHCO_3} = \frac{\lambda C_H}{K}$$

The process of accelerating ventilation and circulation in proportion to the fall in plasma bicarbonate, so that the ratio $\frac{H_2CO_3}{NaHCO_3}$ and the resulting C_H are kept constant, can apparently continue until acidosis is so intense that the respiratory and circulatory mechanisms are no longer able to eliminate carbonic acid so rapidly as to keep its concentration down to one-twentieth that of the depleted bicarbonate. The level to which the bicarbonate falls before this failure of compensation occurs must vary with the sensitiveness of the nervous control and the efficiency of the respiratory and circulatory mechanisms, and has never been definitely fixed, although in diabetes and nephritis it appears to be a small fraction of the normal (Michaelis, 1914, p. 105; Peabody, 1914).

To distinguish the stage of acidosis in which the respiratory mechanism no longer keeps the carbonic acid concentration of the arterial blood down to the normal fraction of approximately one-twentieth the bicarbonate, and in which consequently the C_H actually does increase, Hasselbalch and Gammeltoft

[3] The average C_H is approximately 0.35×10^{-7}. According to Michaelis and Rona (1912), $K = 4.4 \times 10^{-7}$, λ for blood conditions = 0.605. From these constants,

$$\frac{H_2CO_3}{NaHCO_3} = \frac{0.605 \times 0.35 \times 10^{-7}}{4.4 \times 10^{-7}} = \frac{1}{21}$$

(1915) have already used the term "*uncompensated acidosis*," which seems well worth general adoption. So long, on the other hand, as the respiration, despite decreased bicarbonate, succeeds in keeping down to normal limits the $\frac{H_2CO_3}{NaHCO_3}$ ratio, and consequently the C_H, the condition is one of *compensated acidosis*. We shall in future use this nomenclature.

A maintenance of the $\frac{H_2CO_3}{NaHCO_3}$ ratio at a constant value can, of course, be expected only in arterial blood. The H_2CO_3 of venous blood is increased by absorption of CO_2 from the tissues. Consequently venous blood is less alkaline than arterial, and the difference must vary according to the activity with which the tissues perfused are producing carbon dioxide. As was shown by Zuntz (1868), the influx of CO_2 raises not only the H_2CO_3, but by reactions such as $Na_2HPO_4 + H_2CO_3 \rightleftharpoons NaH_2PO_4 + NaHCO_3$, also raises the $NaHCO_3$. Consequently the *arterial* blood bicarbonate must be accepted as the ideal measure of the alkaline reserve. In resting dogs, however, and therefore, it seems justifiable to conclude, in man, the differences between venous and arterial blood are small and fairly constant, the following being fair examples: arterial pH = 7.44, venous 7.41; arterial $NaHCO_3 + H_2CO_3 = 50$ cc. of CO_2 per 100 cc. of blood, venous = 55 cc. The differences are such that analyses of normal venous blood drawn during rest and without stasis may be regarded as but slightly inferior in accuracy and significance to those of arterial blood.

The sense in which we have used the word "acidosis" is not that given it by its originator, Naunyn (1906), who used the term to denote the abnormal metabolic condition in which hydroxybutyric acid is formed. The departure from this use in the literature has been a matter of evolution. Apparently because the word "acidosis" is suggestive of acids in general, rather than hydroxybutyric in particular, when other types of acid intoxication were discovered they also were designated as acidoses. In this broader sense the term has in recent years been used in most of the important scientific papers in the field (for example, Henderson, 1909; Palmer and Henderson, a series of papers; Barcroft, 1914; Sellards, 1914; Peabody, 1914; Howland and Marriott,

1916; Hasselbalch, 1916) "acidosis" being employed to indicate the effect of acids of any type in altering the acid-base balance of the organism. We have followed these authors, rather than those who maintain the original hydroxybutyric acid definition of Naunyn. Despite the value of Naunyn's great work, it appears probable that the confused ideas of acidosis and acid intoxication that have been general have been to a considerable degree due to his definition of the term, which does not differentiate ketone production, unaccompanied by significant effect on the acid-base balance of the body, from the condition in which the acids produced do lower or abolish the reserve of alkali.

The formation of acetone bodies has a significance of its own quite apart from the secondary effect which may or may not follow on the alkaline reserve of the body. It indicates that fatty acids, derived either from fats or from amino-acids, are being incompletely oxidized. The products, β-hydroxybutyric and acetoacetic acids, may or may not be so produced and eliminated that they lower the internal alkaline reserve. We have observed an excretion of 20 gm. of acetone bodies, calculated as hydroxybutyric, per liter of urine without an abnormally low plasma bicarbonate. It is desirable that the condition in which these substances are produced be designated by a name indicating the specific nature of the metabolic abnormality and not confusing it with the general question of the acid-base balance. Rowntree proposes that the excretion of acetone bodies be indicated simply as "ketonuria," while Allen (1917) suggests for the metabolic condition which gives rise to them the equally concise and specific name of "ketosis."

II. Other Methods for Detection of Acidosis Considered as Means for the Approximate Measurement of the Arterial Blood Bicarbonate.

Most methods which have in the past demonstrated some degree of quantitative accuracy in indicating the clinical severity of acidosis are seen when analyzed to constitute approximate determinations, either direct or indirect, of the blood bicarbonate. The following are important examples.

a. Determinations Directly in the Blood.

1. Titration of Blood.—Titration of the blood plasma or of the filtrate obtained after precipitating the proteins with a neutral reagent is one of the oldest methods used in the study of acidosis. The source of error lies in the fact that at the high C_H of the end-points usually employed the titrations measure, in addition to the bicarbonate, also an acid binding power of such buffers as the phosphates, and particularly the proteins, quite out of proportion to the amounts of acid which these substances bind within the C_H limits that occur in the blood during life. Nevertheless, the bicarbonate seems to be the chief cause of variations in the titration figures, and results obtained by this method have consequently been of definite value in developing a knowledge of the changes that constitute acidosis (Jaksch, 1888; Magnus-Levy, 1899; Cullen, Paper III of this series).

2. Determination of the Carbon Dioxide Content of Venous Blood.—The use of the carbon dioxide content (CO_2 from H_2CO_3 and $NaHCO_3$) as a measure of the blood alkali dates back to Walter (1877), who, working in Schmiedeberg's laboratory, showed that the venous carbon dioxide of rabbits could be reduced to one-tenth its normal height by injection of acids. The significance of the determination does not differ essentially from that of the bicarbonate of the venous plasma, determined as described in this paper. In so far as the results indicate the bicarbonate of arterial blood, which must be considered as the true or compensated blood bicarbonate, the source of error lies in the fact that the blood in passing through the capillaries into the veins takes up an amount of carbonic acid which is variable with the rate of oxidation in the tissues and of blood flow through them. As a matter of experience, however, when the blood is drawn from a large vein without stasis, the difference between the venous blood and arterial appears to be sufficiently constant so that the figures for venous total carbon dioxide run approximately parallel to those for the arterial bicarbonate. The failure of Walter's method for detecting acidosis by determination of the venous CO_2 to attain general clinical use even in hospitals must be attributed chiefly to the lack of a sufficiently simple technique for the determination.

3. Determination of the Carbon Dioxide Capacity of Venous Blood.—In order to restore the venous blood to the condition of arterial and thus avoid the possibility of the error outlined in the above paragraph, Christiansen, Douglas, and Haldane (1914) saturated the venous blood with air containing carbon dioxide under the tension existing in normal arterial blood. As illustrated by Experiment IX, the fall caused by acidosis in the carbon dioxide capacity of venous blood is proportional to, and therefore a measure of, the fall in arterial bicarbonate.

In choosing the routine method described in this paper for measuring the alkaline reserve we have given preference to the CO_2 capacity of the *plasma*, rather than either the CO_2 content or the CO_2 capacity of venous whole blood, for practical reasons stated in the discussion of Experiment IX.

4. Determination of the Reduced Hydrogen Ion Concentration of the Blood.—Hasselbalch (1916, *a*) saturated blood with carbon dioxide at 37° under 40 mm. tension and determined under these conditions the C_H, which he calls the "reduced hydrogen ion concentration." The H_2CO_3 concentration being fixed by the constancy of the CO_2 tension and temperature, the hydrogen ion concentration determined must vary inversely as the $NaHCO_3$, since $C_H = K\frac{H_2CO_3}{NaHCO_3}$. Hasselbalch's "reduced hydrogen ion concentration" is therefore a measure of the blood bicarbonate.

5. Determination of the Oxygen Affinity of Hemoglobin under Standard CO_2 Tension.—Barcroft and Peters (Barcroft, 1914, p. 316) found that under changing CO_2 tension the proportion of oxygen bound by hemoglobin depended on the hydrogen ion concentration. The value of the oxygen affinity constant, K, in the equation $\frac{y}{100} = \frac{Kx^n}{1 + Kx^n}$ (y = percentage saturation of hemoglobin with oxygen, x = oxygen pressure) varies inversely as C_H. The results of Barcroft and Peters have been confirmed by Hasselbalch (1916, *b*). Since under a given CO_2 tension the C_H varies inversely as the $NaHCO_3$, it is evident that the oxygen affinity under a given CO_2 tension is also an indirect measure of the bicarbonate; the $NaHCO_3$ fixes the C_H, and through it the oxygen affinity.

b. Determinations on the Alveolar Air.

1. Arterial Carbon Dioxide Tension (Haldane Method).—The alveolar air, as shown by A. and M. Krogh (1910) is in equilibrium in respect to its carbon dioxide content with the arterial blood. Consequently, in accordance with the law of gas solubility, the concentration of carbon dioxide in the alveolar air is directly proportional to that of free carbonic acid in the blood. And the latter has been shown (p. 293), with normal respiratory control, to be kept proportional to the bicarbonate concentration. Consequently the carbon dioxide concentration of the alveolar air is, through the intermediary parallelism of the blood H_2CO_3, kept proportional to arterial $NaHCO_3$. All three concentrations go up and down together, the blood bicarbonate fixing the level of the carbonic acid, and the latter that of the alveolar carbon dioxide. Consequently in normal individuals the Haldane determination of the carbon dioxide content of air expired without previous holding of breath (Haldane and Priestley, 1905) indicates approximately the bicarbonate concentration of the arterial blood. Under pathological conditions, or under the influence of drugs, of decreased atmospheric oxygen tension, or of anxiety or excitement, the sensitiveness of the respiratory control may vary (Hasselbalch, 1912; Michaelis, 1914, p. 97; Higgins, 1915; Straub, 1915; Peters, 1917; Stillman, Van Slyke, Cullen, and Fitz, 1917), so that the alveolar carbon dioxide is not under all conditions even an approximate measure of the bicarbonate reserve. Higgins (1914) found that even changing the position of the body from standing to lying could alter the alveolar CO_2 tension to the extent of 6 mm. Sonne (1915) has shown that a mechanical error may be added to those caused by changes in the nervous control. The air collected at the end of an expiration may fail to represent the average alveolar air, instances in a normal subject being observed in which its CO_2 content was as much as 1.4 volume per cent (corresponding to 10.6 mm. tension, or one-fourth the total normal value) *lower* than the CO_2 content of samples of air taken near the middle of the expiration. Apparently the completeness of the gas exchange varies in different parts of the lungs. All sources of error together, however, even in pathological conditions, are in most of the cases encountered within such limits that the clin-

ical utility of carbon dioxide determinations in the alveolar air as a measure of the alkaline reserve of the blood is thoroughly established (Beddard, Pembrey, and Spriggs, 1915; Straub, 1915); although the fact that so many factors besides the alkaline reserve of the blood can affect the alveolar carbon dioxide tension certainly makes the latter far from an ideal measure of the former.

2. Venous Carbon Dioxide (Plesch Method.)—The Plesch method (Plesch, 1909; Porges and Leimdörfer, 1915) differs from the Haldane in that the air analyzed, instead of being taken at the end of a single quick expiration, is breathed in and out of a rubber bag by the subject for 30 or 40 seconds. Consequently the carbon dioxide tension approaches more nearly that of the venous than of the arterial blood, the Plesch results being as a rule 4 to 6 mm. higher in carbon dioxide tension than the Haldane results. Since the venous carbon dioxide tension runs fairly parallel with the arterial, however, the Plesch results may be taken as indirect measures of the arterial bicarbonate, subject to the same errors as the Haldane results, and so to say, one degree less direct than the Haldane. An advantage of the Plesch technique is that it requires less cooperation on the part of the subject than the Haldane procedure, and has consequently been employed even with infants (Howland and Marriott, 1916).

c. Determinations in the Urine.

1. Determination of the Acid Excretion.—Since Magnus-Levy's famous paper (1899) showed the significance of β-hydroxybutyric acid as the cause of the acid intoxication in diabetic coma, it has been a matter of common observation that symptoms of acid intoxication in diabetes are usually accompanied by the excretion of large amounts of β-hydroxybutyric acid, along with lesser amounts of acetoacetic, partly as ammonium salts, but also partly as free acids. That in diabetes the excretion of free acid plus ammonia by the kidneys bears a *quantitative* relationship to the blood bicarbonate concentration is demonstrated in the accompanying paper by Fitz and Van Slyke.

2. Alkali Retention.—The extremely practical *alkali retention* test devised independently by Palmer and Henderson (1913) and by Sellards (1914) appears also to be an indirect measure of the bi-

carbonate content of the body fluids, as represented by the plasma. Work by Palmer, which will shortly be published in this Journal, indicates that when the plasma bicarbonate (determined as described in this paper) has reached what may be called the critical level, near the upper extreme of the normal range, urine more alkaline than blood is excreted. The amount of bicarbonate which must be taken into the organism in order to turn the urine alkaline is approximately the amount necessary to raise the bicarbonate concentration of all the body fluids to this level, if the fluids are estimated at 0.7 of the body weight and assumed to equal the plasma in bicarbonate content. The amount of alkali necessary to administer in the retention test appears consequently to be proportional to the margin by which the plasma bicarbonate falls below the critical level at the time of administration, and therefore constitutes an indirect measure of the plasma bicarbonate.

III. The Influence of Free Carbonic Acid Concentration in the Blood on the Plasma Bicarbonate.

The plasma bicarbonate concentration is influenced by the free carbonic acid concentration, both of the whole blood at the time the plasma is separated from the cells, and of the plasma itself at the time the determination is made. The influence is exerted respectively through affecting the distribution of acids and bases between plasma and corpuscles, and through affecting reactions within the plasma itself. Both modes of influence must be considered in connection with any method for determining the concentration of plasma bicarbonate in venous blood, and we shall therefore discuss them from the standpoint of their effects on such determinations.

a. Influence through Effect on Equilibria within the Plasma.—To a minor extent the bicarbonate of the plasma can be affected by the equilibrium between normal carbonate, bicarbonate, and carbonic acid: $2NaHCO_3 \rightleftharpoons Na_2CO_3 + H_2CO_3$. As shown by Bohr, however, the conditions of this equilibrium are such that, with the concentrations of free carbonic acid existing in the plasma during life, the proportion of Na_2CO_3 is from a quantitative standpoint negligible, practically all the alkali not bound by other acid than carbonic being in the form of bicarbonate.

In a 0.155 per cent sodium carbonate solution (about the average carbonate concentration of plasma) at 38°, and with the physiologically normal carbon dioxide tension of 45 mm., Bohr calculated that 99.5 per cent of the sodium carbonate was in the form of bicarbonate, and confirmed the calculation experimentally within the limit of analytical error. Even at 12 mm. CO_2 tension, which is seldom if ever observed in life except in premortal coma, 98 per cent was in the form of bicarbonate. Consequently one can, for quantitative purposes, regard the bicarbonate of the plasma as synonymous with its entire reserve of alkali in excess of that neutralized by acids other than carbonic.

There are other equilibria than that between carbonate and bicarbonate, however, which are more sensitive to changes in H_2CO_3 concentration. If carbon dioxide escapes from a sample of plasma, the latter loses not only free carbonic acid CO_2, but also part of the CO_2 normally combined as bicarbonate, which undergoes partial decomposition by such reversible reactions as $NaHCO_3$ + protein $\rightleftharpoons$ H_2CO_3 + Na proteinate. This reaction of the proteins appears in fact to be the one chiefly responsible for the variation in plasma bicarbonate caused by varying free carbonic acid (see Experiments II and III). (While in the cells the reaction $NaHCO_3 + NaH_2PO_4 \rightleftharpoons Na_2HPO_4 + H_2CO_3$, studied by Henderson (1906), is important, in the plasma the phosphate concentration is too small to affect appreciably the bicarbonate (Greenwald, 1915).) If the H_2CO_3 falls greatly below normal even the reaction $2NaHCO_3 \rightleftharpoons H_2CO_3 + Na_2CO_3$ becomes appreciable. In each of these reactions a decrease in the free carbonic acid results in a shift of the equilibrium from left to right, and consequently in a decrease of the bicarbonate. Thus Jaquet found that at 42.7 mm. CO_2 tension the bicarbonate CO_2 of a normal plasma sample was 63.7 volume per cent, while at 17 mm. it was 58.5 per cent. The difference, though not great, is considerable, and becomes accentuated as the carbon dioxide tension is reduced still lower. Consequently in a solution such as the plasma the term bicarbonate content has a quantitatively definite meaning *only for a definite concentration of free carbonic acid.*

One has the choice of two alternatives. One may vary the free carbonic acid in proportion to the bicarbonate, maintaining the 1 : 20 ratio, and thus determining the genuine "compensated" bicarbonate which exists in the arterial blood. Or one may make all determinations at a fixed and definite carbonic acid concentra-

tion. The first plan has the theoretical advantage of duplicating natural conditions, but is impracticable for a routine method, as it would necessitate the use of a different carbon dioxide mixture in saturating every plasma. We have therefore, in the method described in this paper, adopted the plan of saturating all plasmas with carbon dioxide under normal alveolar tension. This has the theoretical disadvantage that in extreme acidosis the bicarbonate determined is not quite so low as that actually existing in the arterial blood. The fall below normal, however, is parallel to that of the arterial bicarbonate (see Experiment IX) and, as a matter of fact, the absolute difference between results by the two methods is not great. For example, the plasma of a diabetic patient with marked acidosis showed, when saturated with CO_2 at the reduced alveolar CO_2 tension of the patient, a bicarbonate yielding 23 cc. of CO_2 per 100 cc. of plasma, while the figure obtained after saturating with CO_2 under normal tension was 26 cc. We believe that under the conditions of constant CO_2 tension chosen the results are no less definite in their significance than they would be if we attempted to approximate the varying CO_2 tension existing in arterial blood.

b. Influence of Carbonic Acid on the Plasma Bicarbonate through Effect on the Transfer of Electrolytes between Plasma and Cells.—Gürber (1895) noticed that as the result of saturating the blood with carbon dioxide *in vitro* the titratable alkali of the plasma, which includes the bicarbonate, was increased. This phenomenon could be explained by assuming either that alkali diffuses from cells into plasma to meet the increased carbonic acid there, or that acids other than carbonic are, so to say, forced by the carbonic from the plasma into the cells, leaving in the form of bicarbonates the alkali with which they had been combined. Gürber claimed that no potassium or sodium at all passed from corpuscles into plasma when blood was saturated with carbon dioxide, that the entire change was due to passage of HCl from the plasma into the cells, the amount which passed being equivalent to the gain in titratable alkali in the plasma. As shown by Experiment X, however, the amount of HCl which disappears from plasma of blood saturated with pure CO_2 is equivalent only to about one-third the increase in bicarbonate. Hamburger (1916) has recently shown, furthermore, there is some transfer of K and Na

between plasma and cells. Consequently Gürber's belief that transfer of HCl alone was responsible for the alkali shift caused by saturating blood with CO_2 does not hold. That the alterations which occur within physiological limits of CO_2 tensions are chiefly due to transfer of HCl appears probable, however (see Experiment X). The reaction in the plasma, the consequent HCl transfer, and reaction of the transferred acid with phosphates inside the cell may be formulated as follows:

$$\underbrace{H_2CO_3 + NaCl \rightleftarrows NaHCO_3 + HCl}_{\text{Plasma}} \xrightarrow[\text{Cell wall}]{HCl\rightarrow} \underbrace{HCl + Na_2HPO_4 \rightleftarrows NaH_2PO_4 + NaHCO_3}_{\text{Cell}}$$

i.e., although the buffer salts may not readily pass out to neutralize acid in the plasma, the acid does pass in to meet the salts within the cells, so that the same effect is obtained in maintaining plasma neutrality. In fact Hamburger, to whose thorough researches we owe most of our knowledge in this field, has shown that it is highly probable that the corpuscles only typify the body cells in general, and that the transfer of acid to and from the latter is of such a nature that the plasma has practically all the buffer salts of the body at its disposal in maintaining its neutrality, despite the fact that it itself is not particularly rich in such salts. In confirmation of this view see Section 2 of the discussion of the results of Experiment IX.

The magnitude of the effect on plasma bicarbonate which can be caused by such loss of carbon dioxide as may occur when blood is drawn into an open receptacle may be seen by reference to Experiment VII, while the extreme effects obtainable, on the one hand by removing CO_2 as completely as possible, on the other by saturating the blood with pure CO_2 gas, are shown by Experiment X.

It is evident that in fixing conditions for the determination of the plasma bicarbonate as a measure of acidosis the concentration of free carbonic acid not only in the plasma at the time of the determination, *but also in the whole blood at the time the corpuscles are separated* from the plasma, must be considered.

The ideal determination would be made on arterial blood drawn without loss of CO_2, for only in the arterial blood is the constancy of the $\frac{H_2CO_3}{NaHCO_3}$ ratio exactly maintained. The use of venous

blood from human subjects being necessary, however, the nearest approximation to arterial conditions would be obtained by saturating the blood at body temperature with CO_2 at such a tension that the normal value of the $\frac{H_2CO_3}{NaHCO_3}$ ratio in the blood would be maintained. This can be approximated by saturating the blood at body temperature with the alveolar air of the subject. In clinical routine, however, such a procedure adds to the technique a complicating factor which our experience indicates is unnecessary. An alternative would be to determine the "carbon dioxide capacity" of the blood after saturating the whole blood with carbon dioxide at the average normal alveolar tension. This method and the practical drawback to its routine application are discussed on pp. 298 and 308. It proved in fact to be entirely practicable to centrifuge the blood as it was obtained directly from the arm vein.

Method for Determining the Plasma Bicarbonate under Constant Dioxide Tension.

1. Drawing Blood Sample—For at least an hour before the blood is drawn the subject should avoid vigorous muscular exertion, as this, presumably because of the lactic acid formed, lowers the bicarbonate of the blood (Christiansen, Douglas, and Haldane, 1914; Morawitz and Walker, 1914). The blood is drawn from the arm vein directly into a centrifuge tube containing enough powdered potassium oxalate to make about 0.5 per cent the weight of the blood drawn. In order to avoid accumulation of carbon dioxide and consequent effect on the electrolyte transfer between plasma and cells, it is desirable to avoid stasis, or when stasis is necessary, to release the ligature as soon as the vein is entered and allow a few seconds for the stagnant blood to pass. This is particularly important when the second procedure for collecting blood described below is used, for in this case no opportunity is given for excess of free CO_2 to escape.

For drawing the blood we have used two methods. For clinical purposes the McRae needle[4] was chiefly employed

[4] The McRae blood needle may be obtained from the Kny-Scheerer or the Tiemann Company of New York. The principle is similar to that of the Hallion and Bauer needle, described in *Compt. rend. Soc. biol.*, 1912, lxxii, 232.

(first method). With this, the blood enters the collecting tube in a fine stream and falls through a height of several centimeters before it reaches the bottom. During this fall there is opportunity for the escape of carbon dioxide and for absorption of oxygen. The carbon dioxide loss, by its effect on the transfer of HCl between plasma and corpuscles, discussed in the preceding pages, measurably lowers the bicarbonate content of the plasma. As a matter of experience, however, the gas exchange which occurs in the interval of about 0.01 second during which the blood is falling is only enough to bring the carbonic acid content of the venous blood to approximately that of arterial (see Experiment VII). The tube is turned on its side and back to vertical position once or twice after the sample has been drawn, in order to mix the oxalate. *The blood is subjected to no other agitation* which might accelerate loss of carbon dioxide, and is centrifuged in the same tube within a few minutes after it has been drawn (for effect of standing see Table X). The results of Stillman, Van Slyke, Cullen, and Fitz (Paper VI of this series), who compared the CO_2 capacity of plasma drawn by this technique with the CO_2 capacity of the whole blood, indicate that the McRae tube can be used in routine clinical work, provided the above precautions are observed, without fear of error.

The other method is to avoid all loss of carbon dioxide and obtain strictly venous blood. For this purpose ordinary care in the use of a syringe is sufficient, the blood being drawn without suction, and free air space in the syringe being avoided. A satisfactory substitute for the syringe is shown in Fig. 1. The blood is collected and centrifuged under paraffin oil. The slight amount of agitation necessary in order to assure mixture of the oxalate is accomplished by stirring with the inlet tube, rather than by inverting or shaking. The paraffin oil, like most organic liquids, dissolves carbon dioxide in greater amounts than does water, and its action in preventing loss of carbon dioxide from the blood is due to prevention of free diffusion from the surface of the water rather than to the formation of a layer impermeable to gas. Consequently the tube is subjected to a minimum of agitation after the blood is in it. When this precaution is taken, the results are the same as those from blood drawn with a syringe (see Experiment VII, *d*).

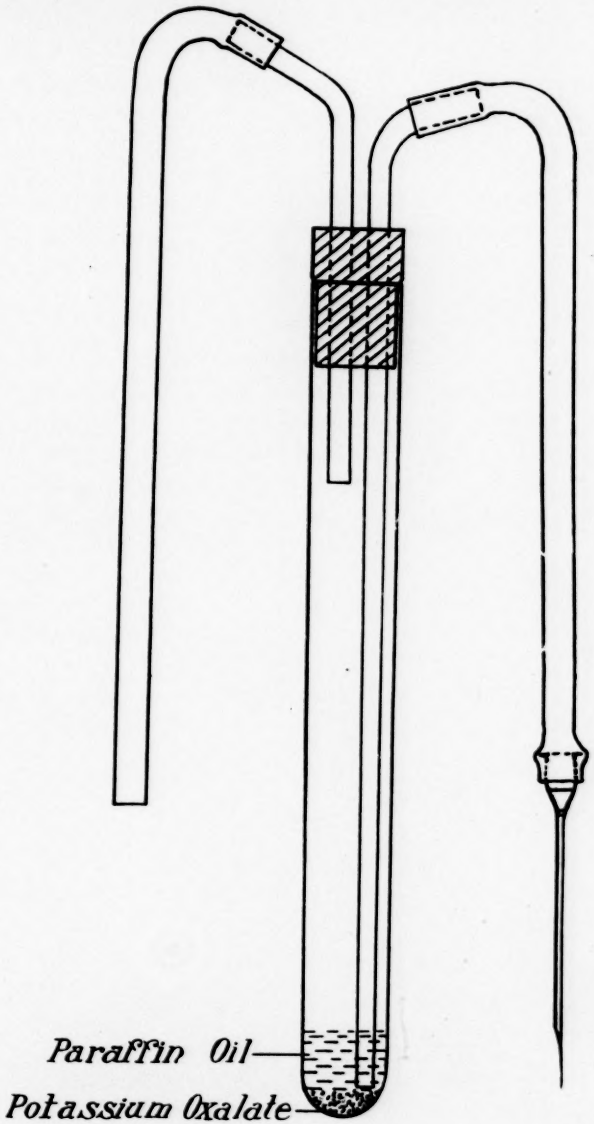

FIG. 1. Centrifuge tube arranged for collecting blood under paraffin oil without loss of carbon dioxide.

When blood is drawn with momentary exposure to air (McRae needle) and analyzed in the manner described in this paper, the plasma being resaturated with air containing 5.5 per cent of CO_2, results of our own and the analyses of thirty normal bloods by Gettler and Baker (1916) show that the bicarbonate CO_2 yielded by 100 cc. of normal plasma varies from 53 to 75 cc., reduced to 0°, 760 mm. A great majority of plasmas show figures between 60 and 70 cc. Occasionally an alkaline diet may force the figure to the upper limit of 80 cc., but we have not yet seen it below 53 in any normal person.

With the second method (venous blood drawn under oil without loss of CO_2) the results of Austin and Jonas (1917) agree with the relatively small number of determinations on human subjects

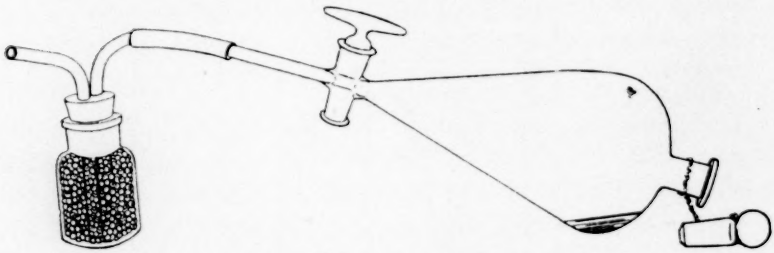

FIG. 2. Separatory funnel containing plasma and arranged for filling with alveolar air.

which we have made in placing the minimum normal figure at 60 instead of 53 cc. of CO_2 per 100 cc. of plasma. With either procedure, but especially with this one, where there is no opportunity for the escape of excess CO_2, the remarks in the first paragraph of this section concerning the avoidance of stagnation are pertinent.

If the difference in the level of the minimum normal values is kept in mind, it appears that the two methods of blood drawing may be used interchangeably (see, for example, Experiment VII, *e*). In this hospital we have used chiefly the McRae needle, but Austin and Jonas, to whom we have communicated both methods and who have tried both, prefer the oil tube.

II. Separation and Storage of Plasma for Analysis.—While results of similar significance are obtained by analysis of either

whole blood or plasma, we prefer the plasma for routine determinations for the following reasons. It can be measured and handled with greater convenience than whole blood. The oxygen bound by the hemoglobin does not complicate the determination when plasma is used. And, perhaps the most important reason, plasma can be kept for a long time without alteration in its carbon dioxide binding capacity, while whole or defibrinated blood begins to show a decrease in its alkaline reserve soon after it has been drawn. This apparent formation of acid in blood in which the corpuscles remain was discovered by Christiansen, Douglas, and Haldane, who found that at 37°, even within an hour after blood had been drawn and defibrinated, an appreciable fall in the carbon dioxide capacity occurs, so that they were able to obtain comparable results only when a constant interval, as short as possible, was allowed to elapse between the drawing of the samples and the determination of their carbon dioxide capacities. The change observed can be most readily explained as due to the formation of acids in the blood cells. The change certainly does not occur in their absence, for we find that sterile plasma, if kept cold and in tubes that have been paraffined in order to avoid solution of alkali from the glass, can be preserved for over a week without alteration in its carbon dioxide capacity. It may be well to state here, however, that plasma in ordinary glass can be kept for only a few hours, as sufficient alkali dissolves from the glass in longer intervals to increase measurably the carbon dioxide capacity.

In case it is necessary to separate the plasma by gravity, the sedimentation is allowed to occur in a closed tube which is completely filled with blood, so that no carbon dioxide can escape, and the plasma is drawn off in as short a time as possible.

Since during rest and normal circulation the carbon dioxide content of the venous blood is only a few per cent higher than that of the arterial, and the difference does not vary greatly, the plasma obtained without stasis might be analyzed at once and without further preparation. As a matter of routine, however, we have found it desirable to saturate the plasma with carbon dioxide at a definite tension, as described below, immediately before analysis, and thus avoid the possibility of error caused by loss of carbon dioxide while the sample is awaiting analysis.

III. Saturation of Plasma with Air Containing Carbon Dioxide under Normal Alveolar Tension.—For the saturation we have found the most convenient vessels to be ordinary separatory funnels capable of holding about 100 times the volume of the plasma that is to be saturated. The plasma is placed in the funnel, the latter is turned on its side, and the air within is displaced by either alveolar air from the lungs of the operator or with 5.5 per cent CO_2-air mixture from a tank. In either case the gas mixture must be passed over glass beads before it enters the funnel (see Fig. 1). Otherwise, when air from the lungs is used the plasma is appreciably diluted with the moisture which condenses from the breath on the inner walls of the funnel. By passage over a large surface of either wet or dry glass beads at room temperature the expired air is cooled, and the excess moisture in it is condensed, so that not enough is carried into the funnel to cause an appreciable error. When, on the contrary, a dry CO_2-air mixture from a tank is used, it causes an appreciable evaporation from the surface of the plasma, with consequent increase in its concentration and in the carbon dioxide capacity. This also is obviated if the gas mixture is passed over wet beads, so that it approaches saturation with water vapor.

For obtaining an artificial mixture of air containing 5.5 per cent of CO_2 we have used an ordinary metallic gas tank capable of standing 20 atmospheres pressure and provided with an accurate pressure gage. Carbon dioxide was run in from another tank until the desired pressure was indicated. Then air was run in until the total pressure of air plus CO_2 was 18.2 times that of the CO_2 (taking into account that the tank contains one atmosphere more than the gage registers). The tank was then laid on its side for a half hour to give the gases an opportunity to mix thoroughly, and samples were drawn for analysis before the mixture was used. The analysis had to be repeated every few days, as the CO_2 content of the gas sometimes changes unexplainably. In order to displace completely the air in the separatory funnel with the CO_2 mixture, five or more volumes were run through, the gas, after leaving the funnel, being collected in a gasometer or rubber bag so that the volume passed could be roughly estimated.

When alveolar air is used, the operator, without inspiring more deeply than normal, expires as quickly and as completely as possible through the bottle of glass beads and the separatory funnel connected as shown in Fig. 2. The stopper is inserted just before the expiration is finished, so that there is no opportunity for air to be drawn back into the funnel. With a little practice a normal

person can consistently fill a 300 cc. separatory funnel with air containing within a few tenths of a per cent of the desired 5.5 per cent of CO_2. The composition is not, of course, so constant as that obtained when an analyzed gas mixture is used to fill the funnel, but as a matter of experience we have never found that the deviations caused significant error in the results. A change of 0.5 per cent in the CO_2 concentration of the air with which the plasma is shaken causes a change of only about 1 volume per cent in the plasma in the amount of CO_2 gas taken up, of which the total is normally 60 to 80 volumes per cent.

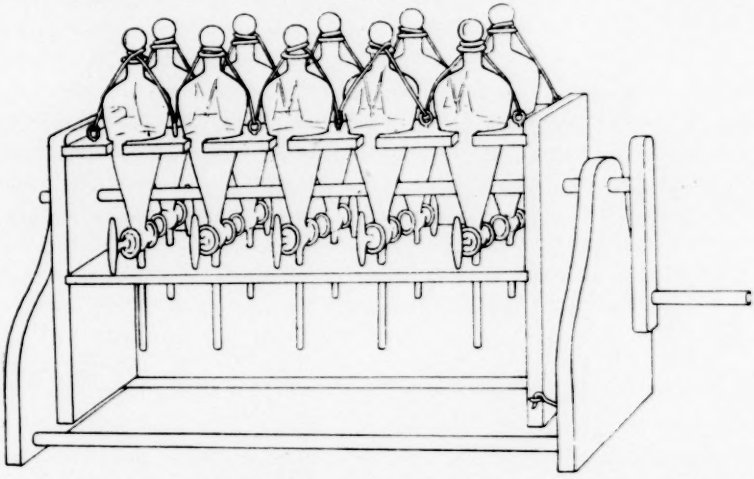

FIG. 3. Rack for holding separatory funnels during saturation of plasma. The elastic cord which holds the stoppers, as well as the entire funnels, in place, is made of spiral wire.

The following figures exemplify the effect of CO_2 concentration in the air on the amount of carbon dioxide taken up by plasma. Samples of the same plasma were shaken in atmospheres of air containing respectively 3.2, 5.5, and 9.6 per cent of carbon dioxide. The results were:

Volume per cent of CO_2 in air.	Gas obtained from 1 cc. of plasma.	Change in absorbed CO_2 due to 1 per cent change in CO_2 of air.
per cent	*cc.*	*cc.*
3.2	0.584	
		0.023
5.5	0.636	
		0.016
9.6	0.700	

In order to saturate the plasma the separatory funnel is turned end over end for 2 minutes, the plasma being distributed in a thin layer as completely over the surface of the funnel's interior as is possible. We have found that 2 minutes' shaking in this manner uniformly suffices for saturation, but that 1 minute is as a rule not enough. When there are several analyses to be done it is convenient to use a rotating rack such as is shown in Fig. 3. In this ten separatory funnels can be shaken at once, and the rack acts as a holder for them at other times.

As a rule, when plenty of plasma is available, we saturate 3 cc. of it in a 300 cc. separatory funnel. One then has sufficient for duplicate determinations on 1 cc. each. As it is possible even with the large apparatus to make a determination with 0.5 cc. of plasma, one can, when the supply is scanty, saturate a little more than 0.5 cc. in a 50 cc. funnel. In this case the volume of distilled water and acid used to wash the plasma into the apparatus is also halved, so that the total volume of water solution introduced is only 1.25 cc. The volume of gas observed is multiplied by 2 before it is used to calculate the volume per cent of CO_2 bound; *i.e.*, a considerable error would be caused if the CO_2 capacity were first calculated from the observed reading, and the result multiplied by 2 *after* the calculation.

When the micro-apparatus for carbon dioxide determinations described in the next paper is used, one-fifth the above amounts of plasma suffices.

IV. Determination of Carbon Dioxide Content of the Saturated Plasma.—After saturation is completed the funnel is placed upright and allowed to stand a few minutes until the fluid has drained from the walls and gathered in the contracted space at the bottom of the funnel. A sample of 1 or 0.5 cc. for the large apparatus, or 0.2 cc. for the micro-apparatus, is drawn with a calibrated pipette and used for the determination of the carbon dioxide content, which is performed as described in the next paper.

When the plasma is being delivered from the pipette into the cup of the apparatus, the tip of the pipette is held below the surface of the liquid in the cup. If the plasma were allowed to run through the air in a fine stream loss of carbon dioxide would result.

It is convenient to use a small drop (0.02 cc.) of octyl alcohol to prevent foaming of the plasma and emulsification of the mercury.

The gas volume is measured after a single extraction, and the result is calculated, by means of the table on p. 316 into terms of volume per cent of carbon dioxide gas, measured at 760 mm., 0°, which is bound as bicarbonate by the plasma.

The carbon dioxide combining capacity of plasma varies appreciably with the temperature, so that human plasma at 20° binds as bicarbonate approximately 106 per cent as much carbonic acid as at 37°. The extent of the temperature effect is demonstrated and its nature discussed in connection with Experiment IV. After determining the temperature coefficients of a number of plasmas we have been able to introduce the average coefficient into the calculation, so that both saturation and analysis can be performed at room temperature without significantly affecting the constancy or reproducibility of the results.

V. Calculation of Results.—When from plasma, saturated as above described with alveolar air, gases are extracted for analysis one obtains not only the CO_2 bound as bicarbonate and set free by acidification, but also the CO_2 and air physically dissolved by the plasma and water. The gases thus dissolved are, of course, independent of the alkaline reserve, and are subtracted from the total in order that the carbon dioxide bound as bicarbonate may be estimated. The exact amount to be subtracted, which is about 0.10 cc. when 1 cc. of plasma is analyzed, but varies slightly with the room temperature, may be determined by blank analyses, or calculated from the known solubility coefficients of the gases.

a. Determination of Correction for Dissolved Gases by Blank Analysis.—A few cc. of acidulated water are saturated with alveolar air or 5.5 per cent CO_2, as described above, and 1 cc. is analyzed with the same technique used for plasma. The total amount of gas obtained is the "dissolved gas" correction. When subtracted from the volume of gas obtained in a plasma analysis made under similar conditions of temperature and pressure, the difference represents the CO_2 chemically bound as bicarbonate in the plasma. This value is multiplied by the factor given in column C, Table I of the next paper, which both reduces the gas to standard conditions (0°, 760 mm.) and corrects for the 4 or 5

per cent of the total CO_2 not removed from the water by the single extraction.

b. Formula Including Corrections for Temperature and Dissolved Gases. Table for Calculation of Results.—In order to calculate the carbon dioxide chemically bound (as bicarbonate) the basic equation of Paper II must be altered by introducing a term which deducts from the total carbon dioxide content of the plasma the amount dissolved as free carbonic acid. This amount in cc. of carbon dioxide gas per cc. of plasma is equal to $p\,\alpha_{CO_2}$ when the plasma is in equilibrium with air containing p proportion of carbon dioxide by volume, α_{CO_2} being the solubility coefficient of carbon dioxide in water. Introducing this subtraction into our basic equation, the latter becomes

	Cc. of CO_2 chemically bound by 1 cc. of plasma.	Factor for reducing gas volume to standard conditions, 0°, 760 mm.	Observed gas volume in cc.	Cc. of air carried into apparatus dissolved in 2.5 cc. of sclution.
(1)	$x =$	f	$\{ V$	$- (2.5 - p)\,\alpha_{air}$

Cc. of CO_2 kept in solution after first extraction.	Cc. of CO_2 dissolved in 1 cc. plasma as free carbonic acid.
$+ 0.053\,(V - [2.5 - p]\,\alpha_{air})\,\alpha_{CO_2}$	$- 0.975\,p\,\alpha_{CO_2}\}$

The term $(2.5 - p)\,\alpha_{air}$ is derived as follows. The volume of air dissolved in 1 cc. of plasma shaken with air containing p proportion of carbon dioxide is $(1 - p)\,\alpha_{air}$. The volume held in solution under atmospheric pressure by the 1.5 cc. of water and dilute acid used in washing the plasma into the apparatus is $1.5\,\alpha_{air}$. Hence the total correction for the air in the gas volume observed is $(1 - p)\,\alpha_{air} + 1.5$ air, or $(2.5 - p)\,\alpha_{air}$. When p is only 0.055, as is the case when determining the carbon dioxide capacity of plasma in the manner described in this paper, its effect on this term is negligible; but if air containing proportions of carbon dioxide much higher than the physiological 5.5 per cent was utilized in saturating the plasma the effect of p would become measurable. This was the case in some experiments to be reported later, and the value of p is introduced into the equation so that it can be used in such cases.

The derivation of the other terms is self-evident. The last term has the coefficient 0.975 because Bohr has shown that the dissolved substances in plasma reduce the solubility of gases in it to 97.5 per cent of their solubilities in pure water. As in the case of p, this factor, 0.975, exerts an

appreciable influence on the results calculated only when the plasma is saturated with gas containing a much higher percentage of carbon dioxide than the 5.5 per cent used in routine determinations of carbon dioxide capacities.

For routine determinations the equation is reduced to a working basis by substituting 0.055 for p, and by introducing the temperature coefficients for the various constants, in the manner employed in the derivation of Equation 4 of Paper II. We then have

$$x = \frac{B}{760} (107.3 - 0.586\,t)\ (V - 0.136 + 0.002\,t) \tag{2}$$

t being the temperature centigrade.

As will be shortly shown, however, the carbon dioxide combining capacity of plasma decreases by an average of 0.36 per cent for each degree rise in the temperature at which the plasma is saturated with the CO_2-air mixture. In order to have results obtained at different room temperatures accurately comparable, therefore, we have introduced this additional temperature coefficient into the calculation in such a manner that the results calculated indicate the amount of carbon dioxide the plasma would bind if it were saturated at 20°. Introducing the temperature coefficient 0.0036 in this manner, we have

$$x = \frac{B}{760} \frac{107.3 - 0.586\,t}{1 + 0.0036\,(t - 20)} (V - 0.136 + 0.002\,t) \tag{3}$$

The values of

$$\frac{107.3 - 0.586\,t}{1 + 0.0036\,(t - 20)}$$

may be accurately expressed between 15° and 30° by the term 100.8 − 0.27t. Hence the equation becomes

$$x = \frac{B}{760} (100.8 - 0.27\,t)\ (V - 0.136 + 0.002\,t) \tag{4}$$

x expressing the cc. of CO_2 reduced to 0°, 760 mm., which 1 cc. of plasma will bind as bicarbonate when in equilibrium at 20° with air containing 5.5 per cent by volume of carbon dioxide.

TABLE I.

Table for Calculation of Carbon Dioxide Combining Power of Plasma.

Observed vol. gas $\times \frac{B}{760}$.	Cc. of CO_2, reduced to 0°, 760 mm., bound as bicarbonate by 100 cc. of plasma.				Observed vol. gas $\times \frac{B}{760}$.	Cc. of CO_2, reduced to 0°, 760 mm., bound as bicarbonate by 100 cc. of plasma.			
	15°	20°	25°	30°		15°	20°	25°	30°
0.20	9.1	9.9	10.7	11.8	0.60	47.7	48.1	48.5	48.6
1	10.1	10.9	11.7	12.6	1	48.7	49.0	49.4	49.5
2	11.0	11.8	12.6	13.5	2	49.7	50.0	50.4	50.4
3	12.0	12.8	13.6	14.3	3	50.7	51.0	51.3	51.4
4	13.0	13.7	14.5	15.2	4	51.6	51.9	52.2	52.3
5	13.9	14.7	15.5	16.1	5	52.6	52.8	53.2	53.2
6	14.9	15.7	16.4	17.0	6	53.6	53.8	54.1	54.1
7	15.9	16.6	17.4	18.0	7	54.5	54.8	55.1	55.1
8	16.8	17.6	18.3	18.9	8	55.5	55.7	56.0	56.0
9	17.8	18.5	19.2	19.8	9	56.5	56.7	57.0	56.9
0.30	18.8	19.5	20.2	20.8	0.70	57.4	57.6	57.9	57.9
1	19.7	20.4	21.1	21.7	1	58.4	58.6	58.9	58.8
2	20.7	21.4	22.1	22.6	2	59.4	59.5	59.8	59.7
3	21.7	22.3	23.0	23.5	3	60.3	60.5	60.7	60.6
4	22.6	23.3	24.0	24.5	4	61.3	61.4	61.7	61.6
5	23.6	24.2	24.9	25.4	5	62.3	62.4	62.6	62.5
6	24.6	25.2	25.8	26.3	6	63.2	63.3	63.6	63.4
7	25.5	26.2	26.8	27.3	7	64.2	64.3	64.5	64.3
8	26.5	27.1	27.7	28.2	8	65.2	65.3	65.5	65.3
9	27.5	28.1	28.7	29.1	9	66.1	66.2	66.4	66.2
0.40	28.4	29.0	29.6	30.0	0.80	67.1	67.2	67.3	67.1
1	29.4	30.0	30.5	31.0	1	68.1	68.1	68.3	68.0
2	30.3	30.9	31.5	31.9	2	69.0	69.1	69.2	69.0
3	31.3	31.9	32.4	32.8	3	70.0	70.0	70.2	69.9
4	32.3	32.8	33.4	33.8	4	71.0	71.0	71.1	70.8
5	33.2	33.8	34.3	34.7	5	71.9	72.0	72.1	71.8
6	34.2	34.7	35.3	35.6	6	72.9	72.9	73.0	72.7
7	35.2	35.7	36.2	36.5	7	73.9	73.9	74.0	73.6
8	36.1	36.6	37.2	37.4	8	74.8	74.8	74.9	74.5
9	37.1	37.6	38.1	38.4	9	75.8	75.8	75.8	75.4
0.50	38.1	38.5	39.0	39.3	0.90	76.8	76.7	76.8	76.4
1	39.1	39.5	40.0	40.3	1	77.8	77.7	77.7	77.3
2	40.0	40.4	40.9	41.2	2	78.7	78.6	78.7	78.2
3	41.0	41.4	41.9	42.1	3	79.7	79.6	79.6	79.2
4	42.0	42.4	42.8	43.0	4	80.7	80.5	80.6	80.1
5	42.9	43.3	43.8	43.9	5	81.6	81.5	81.5	81.0
6	43.9	44.3	44.7	44.9	6	82.6	82.5	82.4	82.0
7	44.9	45.3	45.7	45.8	7	83.6	83.4	83.4	82.9
8	45.8	46.2	46.6	46.7	8	84.5	84.4	84.3	83.8
9	46.8	47.1	47.5	47.6	9	85.5	85.3	85.2	84.8
0.60	47.7	48.1	48.5	48.6	1.00	86.5	86.2	86.2	85.7

The temperature figures at the heads of columns represent the room temperatures at which the samples of plasma are saturated with alveolar CO_2 and analyzed. It is assumed that both operations are performed at the same temperature. The figures have been so calculated that, regardless of the room temperature at which saturation and analysis are performed, the table gives the volume (reduced to 0°, 760 mm.) of CO_2 that 100 cc. of plasma are capable of binding when saturated at 20° with CO_2 at approximately 41 mm. tension. If the figures in the table are multiplied by 0.94 they give, within 1 or 2 per cent, the CO_2 bound at 37°.

If the figures in the table are multiplied by 0.66 they give the mm. CO_2 tension of the alveolar air (Haldane method) if the relationship between alveolar carbon dioxide and plasma bicarbonate is the *average* normal. The physiological deviations from this average may be as great as 7 mm. (Paper V), the pathological much greater (Paper VI).

For convenience in the calculation the values for the ratio $\frac{\text{barometer}}{760}$ over the range usually encountered are given below.

Barometer.	$\frac{\text{Barometer}}{760}$	Barometer.	$\frac{\text{Barometer}}{760}$
732	0.961	756	0.995
734	0.996	758	0.997
736	0.967	760	1.000
738	0.971	762	1.003
740	0.974	764	1.006
742	0.976	766	1.008
744	0.979	768	1.011
746	0.981	770	1.013
748	0.984	772	1.016
750	0.987	774	1.018
752	0.989	776	1.021
754	0.992	778	1.024

In order to express results in mg. of CO_2 bound by 1 cc. of plasma, the factor $\frac{\text{weight in mg. of 1 cc. } CO_2}{100} = 0.01964$ may be introduced, yielding

(5) Mg. CO_2 bound chemically by 1 cc. plasma

$$= \frac{B}{760}(1.982 - 0.0053\,t)(V - 0.136 + 0.002\,t)$$

In order to avoid the necessity of calculation in routine work, we have computed Table I by means of Equation 4. By means of

this table the readings on the apparatus can be directly transposed into cc. of CO_2 chemically bound by 100 cc. of plasma. It will be seen that when the gas volume reading is above 0.50 cc. the various temperature effects nearly neutralize each other, so that a reading of 0.70 cc., for example, indicates almost exactly the same carbon dioxide capacity, whether saturation and analysis are performed at 15° or at 25°.

EXPERIMENTAL.

I. The Non-Effect of Potassium Oxalate on the Carbon Dioxide Capacity of Plasma.

a. The Effect of Oxalate on the Carbon Dioxide Capacity of Water.—Before oxalate could be used to prevent coagulation of blood it was necessary to demonstrate that its introduction causes no appreciable change in the carbon dioxide capacity. As shown by the following experiment, a solution of potassium oxalate dissolves under given conditions more carbon dioxide than does pure water.

Water and solutions containing respectively 1 and 10 per cent of potassium oxalate were shaken at 29° with air containing 23.0 per cent of carbon dioxide by volume. The carbon dioxide content of the solutions was determined as described in the preceding paper, with the exception that 2 cc. of each solution instead of 1 cc. were taken for analysis. The following results were obtained.

TABLE II

Solution.	Vol. of CO_2 dissolved.	α CO_2 observed.	α CO_2 observed by Bohr and Bock.
Water	0.345	0.75	0.753
1 per cent oxalate	0.435	0.92	—
10 " " "	0.480	1.04	—

It is evident that the oxalate imparts to pure water a slight alkalinity which can be measured by so delicate a means as the carbon dioxide capacity. Pure water is immensely more sensitive to the effects of solutes of slightly acid or alkaline nature, however, than are solutions containing buffers, like carbonates, phosphates, and proteins. Blood is preeminently such a solution,

and in order to make experiments on the effect of oxalate applicable to blood plasma, they must be performed upon solutions which imitate the buffer composition of the plasma.

b. Effect of Oxalate on the Carbon Dioxide Capacity of Phosphate Solutions.—Soerensen's solutions of pure phosphates in M/15 concentration were used. 10 cc. of KH_2PO_4 solution and 40 cc. of Na_2HPO_4 solution were mixed, the resulting solution having a pH of 7.38, approximately that of the blood. The carbon dioxide capacity of this solution was determined in exactly the manner described in the preceding pages for plasma, except that 5.0 per cent CO_2-air mixture instead of 5.5 per cent was used. The following results were obtained.

TABLE III.

Solution used.	Vol. gas observed.	CO_2 dissolved.
	cc.	*mg.*
Phosphate	0.260	0.396
	0.265	0.405
Phosphate + 1 per cent oxalate	0.260	0.396
	0.262	0.400
Phosphate + 10 per cent oxalate	0.260	0.396
	0.262	0.400

The determinations were made at 24°, 760 mm., and the results in mg. calculated by the formula for 24° given in Table I of the succeeding article.

It is evident that oxalate, even up to 10 per cent concentration, does not affect the carbon dioxide capacity of the phosphate solution.

c. Effect of Oxalate on the Carbon Dioxide Capacity of Sodium Carbonate Solution.—A 0.1 per cent solution of sodium carbonate was used for the experiment which in all details was similar to that preceding. The carbon dioxide capacity of this solution is slightly less than that of normal plasma.

TABLE IV.

Solution used.	Vol. gas observed at 24°, 760 mm.	Total CO_2 dissolved.	CO_2 chemically bound.
	cc.	*mg.*	*mg.*
0.1 per cent Na_2CO_3	0.534	0.90	0.826
0.1 per cent Na_2CO_3 + 1 per cent oxalate	0.529	0.89	0.816
0.1 per cent Na_2CO_3 + 10 per cent oxalate	0.515	0.865	0.791

The presence of 1 per cent of oxalate had no effect on the carbon dioxide capacity of 0.1 per cent Na_2CO_3 solution outside the limit of error of the determination. 10 per cent of oxalate did not increase, but reduced the carbon dioxide capacity of the solution detectibly, an effect which may be attributed to the fact that the addition of so much solid oxalate to the carbonate solution appreciably increased its volume, so that it contained less than 1 mg. of Na_2CO_3 per cc. To a minor degree the effect is also due to the well known fact that the presence of salts reduces the solubility of gases in water, so that less CO_2 is dissolved as free H_2CO_3 than in pure water solution.

The results of both preceding experiments show that potassium oxalate in 1 per cent and even greater concentration does not affect the carbon dioxide capacities of solutions containing concentrations of phosphate or of sodium carbonate such as would bind the amounts of carbon dioxide held by the plasma. Consequently the oxalate appears to be excluded as a source of error in our determinations on plasma.

It may be noted that the amounts of CO_2 bound as carbonate, 0.826 mg. in absence of oxalate, 0.816 mg. in the presence of 1 per cent oxalate, are very near the amount, 0.830 mg., that must be bound to convert all the carbonate into $NaHCO_3$. The results harmonize with those of Bohr, who determined both by calculation and by analysis that nearly 100 per cent of the sodium carbonate in the presence of free carbonic acid at alveolar tension must be in the form of bicarbonate.

d. Comparison of Oxalate and Hirudin Plasmas.—Blood from an arm vein was drawn into a tube containing a few flakes of hirudin, and the carbon dioxide capacity of the plasma was determined. Duplicate readings were 0.74 and 0.74 cc. of gas at 23°, 760 mm., indicating a capacity of 61.6 volume per cent of carbon dioxide bound by 1 cc. of plasma. In 5 cc. of the plasma 0.050 gm. (1 per cent) of potassium oxalate was dissolved, and the determinations were repeated. The readings were again 0.74 and 0.74 cc., showing that the oxalate had no measurable effect on the results.

This experiment is final proof that even twice as much oxalate as is used in our routine is without significant effect on the carbon dioxide capacity of the plasma.

Experiment II. Effect of Concentration of Free Carbonic Acid on the Amount Bound as Bicarbonate by Plasma.—In order to obtain evidence concerning the magnitude of the effect which changes in the carbon dioxide content of the air used for saturating would have on results of our plasma analyses, we have determined the amounts of carbon dioxide absorbed by plasma in equilibrium with atmospheres containing from 3 per cent of carbon dioxide upwards. The results are given in Table V and Fig. 4.

The plasma was a mixture of several samples from diabetic patients, some of whom showed slight degrees of acidosis. The mixture of plasmas showed about the minimum carbon dioxide capacity which we have observed in plasma from normal individuals. The determinations of carbon dioxide were performed on 1 cc. samples in the usual manner. Because of the variation in the carbon dioxide content of the saturating air, however, the results could not be calculated by the table at the end of this paper, but had to be reckoned by direct application of Equation 1 (p. 314). The barometer was 760 mm. and the temperature 27°. Under these conditions the constants of Equation 1 have the following values: $f = 0.827$; $\alpha_{CO_2} = 0.79$; $\alpha_{air} = 0.0165$.

Experiment III. Effect of Free Carbonic Acid on the Amounts Bound by Sodium Carbonate, Sodium Phosphate, and Sodium Albuminate.—These data form but the preliminary steps of a study of the carbon dioxide carrying mechanism of the blood, but they are presented here because they are at least suggestive of the manner in which carbonic acid reacts with the blood constituents.

Sodium Carbonate.—A 0.1 per cent solution (0.0188 N) of Merck's "reagent" Na_2CO_3 was saturated with carbon dioxide under varying tensions. The amounts of bound carbon dioxide were calculated, as in the case of the plasma in the preceding experiment, with the aid of Formula 1, which here, however, is altered by removal of the factor 0.975 from the last term, since such a dilute solution may be assumed to have practically 100 per cent of the dissolving power of water for gases, instead of the 97.5 per cent observed by Bohr in the case of plasma.

The experiment with sodium carbonate serves chiefly the purpose of a control of the methods as employed over a wide range of carbon dioxide tensions. As will be seen from the Na_2CO_3 curve of Fig. 4, the method gave practically theoretical results throughout the entire range of tensions, the Na_2CO_3 binding the amount of H_2CO_3 necessary to convert it into $NaHCO_3$.

Sodium Albuminate.—4 gm. of Merck's egg albumin were dissolved in 25 cc. of water in a 50 cc. flask, and 0.5 cc. of a 1 per cent solution of phenolphthalein was added. A solution of N/7 sodium hydrate was then added from a burette until the albumin assumed the rose-color indicating a hydrogen ion concentration of approximately $10^{-8.5}$, which is about that of plasma from which the free carbonic acid has been pumped out. The solution was then diluted up to the 50 cc. mark. The concentration of

TABLE V.

Effect of Carbonic Acid Concentration on the Amount of CO_2 Bound as Bicarbonate by Plasma.

CO_2 in air used for saturating plasma.	V. Vol. gas extracted from 1 cc. of saturated plasma.	Average V.	CO_2, reduced to 0°, 760 mm., in 100 cc. of plasma.			Molecular concentration of bicarbonate.
			Total.	Dissolved as free carbonic acid.	Bound as bicarbonate.	
per cent	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	
3.2	0.627 0.622	0.625	53.4	2.1	51.3	0.0229
5.5	0.676 0.676	0.676	58.1	3.7	54.4	0.0243
Alveolar air.	0.681 0.691	0.686	59.0	3.7*	55.3	0.0247
9.6	0.740 0.740	0.740	64.0	6.4	57.6	0.0259
23.0	0.898 0.898	0.898	78.4	15.6	62.8	0.0280
100	0.779 0.779 (From 0.5 cc. of plasma.)	0.779	127.0	68.0	69.0	0.0308

* Calculated on the assumption that the alveolar air from the analyst's lungs contained the average normal of 5.5 per cent of carbon dioxide. It may, of course, vary several tenths of a per cent from this.

The apparent agreement of most of the duplicates to within 0.001 cc. on the readings is somewhat misleading. The readings were made to the nearest 0.005 cc., and corrected according to the calibration of the burette. Absolute apparent agreement, therefore, indicates agreement not necessarily closer than 0.005 cc., which is about as close as one can read the instrument with a slightly milky solution like diluted and acidified plasma.

albumin, 8 per cent, was approximately that of the proteins of the plasma. The concentration of sodium in the solution was 0.0143, sufficient to bind as bicarbonate about one-half the amount of carbon dioxide held by normal human plasma.

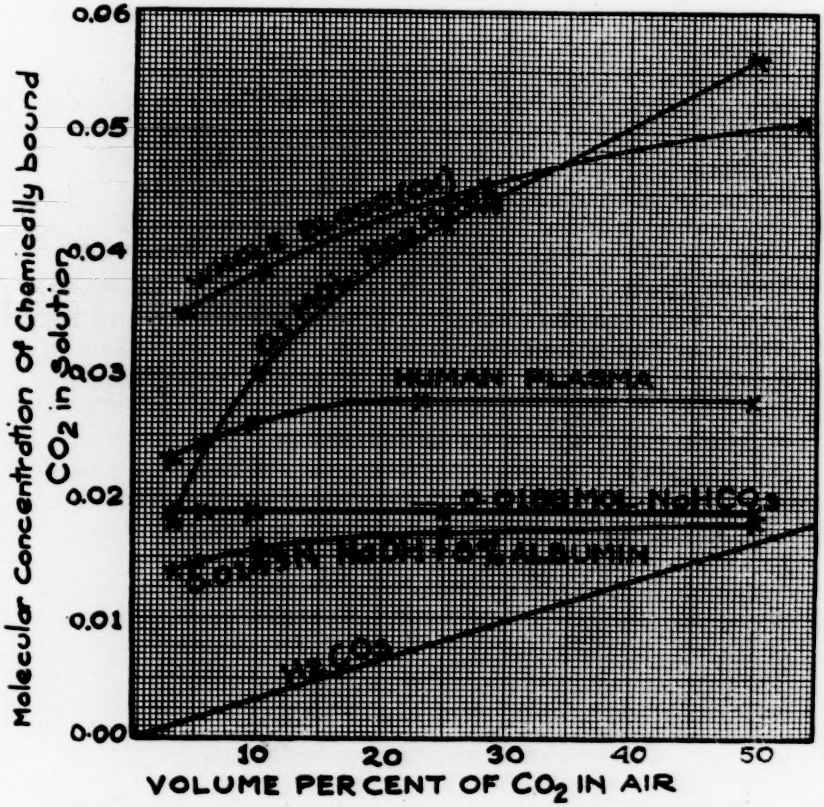

FIG. 4. Effect of free carbonic acid on the amounts bound as bicarbonate by plasma, and by sodium albuminate solution of alkali and protein concentrations similar to those of plasma.

Sodium Phosphate.—A tenth molecular solution of Na_2HPO_4 prepared according to Soerensen was used. The phosphate solutions require for the attainment of equilibrium with atmospheric carbon dioxide considerably more time than the 2 minutes' shaking which suffices for plasma and carbonate solutions. The phosphate solutions were accordingly shaken

repeatedly with the gas mixtures, and analyzed after each shaking, until constant results showed that the maximum amounts of carbon dioxide had been absorbed by the reaction $H_2CO_3 + Na_2HPO_4 = NaHCO_3 + NaH_2PO_4$.

It will be seen that the curve obtained with the albuminate solution closely resembles that obtained with plasma. In equilibrium with 5.5 per cent carbon dioxide in the air the solution binds almost exactly the amount of carbon dioxide that the sodium hydrate alone would combine with if no albumin were present. When the carbonic acid content of the solution is greatly increased the albumin also binds an appreciable though small amount of carbon dioxide, as is evidenced by the fact that more is taken up than the sodium hydrate alone could account for. This does not prove that in the plasma all the carbon dioxide binding power is due to alkaline carbonate and protein,—the plasma curve can also be almost exactly duplicated by a solution containing 0.01 M Na_2HPO_4 and 0.02 M $NaHCO_3$. It is known, however, that the plasma contains only about 3 mg. of inorganic phosphorus per 100 cc. (Greenwald, 1915) which is too little to affect measurably the carbon dioxide capacity. Nor do ash analyses on the plasma indicate the presence of significant amounts of any other crystalloid buffers. The weight of evidence indicates that the only buffers of significance in the plasma are the proteins and the carbonates.

The whole blood, on the other hand, yields a constantly rising curve like the phosphate solution, indicating the participation of the relatively abundant phosphates of the corpuscles.

Experiment IV. Effect of Temperature Saturation on the Amount of Carbon Dioxide Bound by Plasma.

The determinations were made in the usual manner, except that in order to saturate at 10° and 40° the separatory funnels were shaken in baths at these temperatures. Short thermometers dipping into the plasma were placed inside the funnels, and the saturations were finished after the desired temperatures had been reached. Results were obtained with plasma from seven different individuals, and were calculated by means of Equation 1. The values for the solubilities of air and carbon dioxide used in the formula are, of course, partly the values for room temperature, partly for the temperature of saturation. The results are given in Table VI, and in the curves of Fig. 5.

The approximately linear form of the curves of Fig. 5 shows that the temperature effect between 10° and 40° is fairly constant. The maximum percentage decrease in carbon dioxide capacity caused by 1° rise in temperature was 0.47 per cent of the amount of carbon dioxide bound at 20°. The minimum was 0.25 per

TABLE VI.

Effect of Temperature on the Carbon Dioxide Binding Power of Human Plasma.

Plasma No.	Temperature of saturation.	Room temperature.	Barometer.	V. Vol. of gas observed.	CO_2 per 100 cc. plasma.			Decrease in bound CO_2 per 1° increase in temperature between 10° and 40°.	
					Total.	Dissolved as free carbonic acid.	Bound as bicarbonate.		
	°C.	°C.	mm	cc.	cc.	cc.	cc.	cc.	per cent of CO_2 bound at 20°
1	10	24.5	758	0.700	60.6	6.5	54.1		
	24.5	24.5	759	0.650	56.3	4.1	52.2	0.13	0.25
	40	24.5	758	0.605	52.8	2.7	50.1		
2	10	23.5	761	0.855	75.7	6.5	68.2		
	23.5	23.5	761	0.780	69.2	4.2	65.0	0.31	0.47
	40	23.5	761	0.701	62.3	2.7	59.6		
3	10	24	757	0.721	62.5	6.5	56.0		
	24	24	757	0.671	58.4	4.2	54.2	0.14	0.25
	40	24	757	0.623	54.6	2.7	51.9		
4	10	22	754	0.781	68.7	6.5	62.2		
	22	22	754	0.721	63.4	4.5	58.9	0.17	0.28
	40	22	749	0.762	60.0	2.7	57.3		
5	10	24	757	0.791	69.0	6.5	62.5		
	24	24	757	0.711	62.5	4.2	58.3	0.24	0.40
	40	24	757	0.662	58.1	2.7	55.4		
6	10	24	757	0.721	63.6	6.5	57.1		
	24	24	757	0.671	58.6	4.2	54.4	0.14	0.25
	40	24	757	0.623	55.6	2.7	52.9		
7	10	22	754	0.781	69.7	6.5	63.2		
	22	22	754	0.721	63.0	4.5	58.5	0.24	0.40
	40	22	748	0.682	58.9	2.7	56.2		

cent; the mean between the extremes is 0.36 per cent. The variation on both sides of the mean is large compared with the size of the mean value itself. As the entire temperature effect is so small, however, the mean temperature coefficient can be used over the range of ordinary room temperature without introducing significant errors.

The decrease in carbonic acid binding power caused by increase in temperature appears to result chiefly from the lowering of the solubility of carbon dioxide and the consequent decrease in the concentration of free carbonic acid in the experiments at the higher temperatures. Raising the temperature from 10° to 40° diminished the carbonic acid CO_2 from 6.5 volume per cent to 2.7.

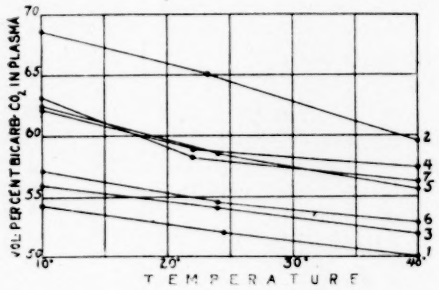

FIG. 5. Effect of temperature on the carbon dioxide capacity of plasma.

The average effect on the bicarbonate CO_2 in the seven plasmas was to lower it 6 volumes per cent. Interpolation on the curve of Fig. 4 indicates that in the experiment there tabulated a lowering of carbonic acid CO_2 from 6.5 to 2.7 volume per cent without any temperature change decreased the plasma bicarbonate 5.1 volume per cent, or nearly as much as when the change in free carbonic acid was accompanied by an increase of 30° in temperature. That temperature does influence the equilibria between carbon dioxide and buffers independently of its effect on carbon dioxide solubility is certain (Henderson, 1906), but the effect in the plasma appears to be slight compared with that of changing concentrations of free carbonic acid.

Experiment V. The Effect of Acids on the Carbon Capacity of Plasma.

β-Hydroxybutyric Acid.—Varying amounts of standardized solutions of Kahlbaum's hydroxybutyric acid were weighed into portions of a sample of human plasma, the concentrations of acid being such that the volume increase caused by its addition to the plasma was always less than 1 per cent. The plasmas were then saturated with 5.5 per cent carbon dioxide and analyzed.

Hydrochloric Acid.—Varying amounts of 0.1 N and 0.2 N HCl were added to 10 cc. portions of another plasma, with sufficient water in each case to bring the volume up to 12 cc. The mixtures were then saturated with 5.5 per cent CO_2 and analyzed.

The results with both acids are given in Table VII.

TABLE VII.

Acid.	Concentration of acid. Mols. per liter.	CO_2 bound as bicarbonate.		Decrease in bicarbonate caused by acid. Mols. per liter.
		Vol. per cent.	Mols. per liter.	
Hydrochloric.	0.0000	58.0	0.0259	0.0000
	0.0042	48.5	0.0216	0.0043
	0.0083	41.3	0.0184	0.0075
	0.0167	25.3	0.0113	0.0146
	0.0250	12.5	0.0056	0.0203
	0.0333	2.8	0.0012	0.0247
	0.0500	0.0	0.0000	
β-Hydroxybutyric.	0.0000	67.6	0.0283	0.0000
	0.0096	43.3	0.0182	0.0101
	0.0240	23.9	0.0100	0.0183
	0.0481	3.7	0.0016	0.0267
	0.0962	0.0	0.0000	

Comparison of the first and last columns of the table shows that until acid equivalent to about half the plasma bicarbonate has been added the fall in bicarbonate approximately equals in molecular equivalents the amount of acid added. As the amount of acid becomes greater, however, the drop in plasma bicarbonate begins to fall short of the added acid. This is due to the fact that the H_2CO_3 concentration is kept constant, instead of being reduced in proportion to the bicarbonate. The condition is similar to that of the blood in uncompensated acidosis. The $\frac{H_2CO_3}{NaHCO_3}$ ratio, and consequently the C_H, is increased. As a result the other

plasma buffers (chiefly proteins) bind a measurably greater amount of acid than they could at normal C_H, and the acid so bound is prevented from decomposing bicarbonate.

The effect on the routine plasma determination is that the bicarbonate determined by our technique denotes a fall in the more severe stages of acidosis which is not quite so great as the actual drop in bicarbonate *in vivo*. The relationship between added acid and decrease in bicarbonate, however, is made so constant by saturating the plasma at a definite carbon dioxide tension that the lack of absolute numerical proportionality in the lower ranges is no practical detriment to the interpretation of results.

Experiment VI. Effect of Preservation on the Carbon Dioxide Capacity of Plasma.

A sample of oxalated human plasma was placed in a paraffin-lined tube in a refrigerator at approximately +1°C. Samples were removed at intervals and analyzed with the following results.

TABLE VIII.

Age of plasma sample kept at + 1° in paraffined tube.	Gas vol. read.	Temperature.	Barometer.	CO_2 capacity. (Cc. of CO_2, reduced to 0°, 760 mm., bound chemically by 100 cc. of plasma.)
days	*cc.*	*°C.*	*mm.*	*vol. per cent*
0	0.70	21	750	56.8
1	0.71	19	750	57.6
2	0.71	21	753	57.8
4	0.70	22	752	57.0
6	0.70	22	752	57.1
12	0.68	27	767	56.7
20	0.61	20	764	49.4

For 12 days the capacity of the plasma remained stationary at 57.3 ±0.5 volume per cent of carbon dioxide. The first change was noted in the 20 day sample, and was probably due to bacteria, as the samples for analysis were not drawn under sterile conditions. It may be well, however, to emphasize the fact that plasma kept in ordinary glass, instead of paraffin, may dissolve enough alkali over night to increase its carbon dioxide capacity by several per cent.

VII. Effects of Manner of Drawing Blood on the Carbon Dioxide Content of Whole Blood Samples, and the Carbon Dioxide Capacity of the Plasma.

Experiment VII, a. Blood from Different Veins Compared with Arterial. Effect of Momentary Exposure to Air.

An 18 kilo dog was etherized with a cone (no artificial respiration) and the femoral artery and vein of the right thigh, the brachial artery and vein of the left fore leg, and the left external jugular vein were exposed. Blood samples were drawn from each vessel both by means of a syringe, and of a needle connected with a 50 cc. Erlenmeyer flask. The latter was fitted with a two-hole rubber stopper through which passed two short glass tubes extending for 1 or 2 cm. both above and below the stopper. To one of the tubes was attached about 10 cm. of small bore rubber tubing, into the farther end of which was fitted the needle. To the other glass tube was attached a somewhat longer piece of rubber tubing, by means of which suction could be applied to accelerate the flow of blood from the vein. As in the case of the McRae needle, the blood was drawn with moderate suction into an open vessel, and fell through a height of several centimeters, so that for 0.01 second or less opportunity was given for escape of venous carbon dioxide.

Blood was allowed to flow into syringes simultaneously from the femoral and jugular veins. Then the syringes were immediately replaced by two collecting flasks of the kind described above, and samples of blood were drawn into them. Consequently these four samples may be considered as practically simultaneous.

The same procedure was then repeated with the femoral artery, brachial vein, and brachial artery. The entire operation, from beginning of anesthetization through the drawing of the second set of samples, consumed 30 minutes. Samples of the whole blood were analyzed for carbon dioxide content at once, the technique described on p. 361, of the succeeding article being employed. Other samples were meanwhile centrifuged, and the carbon dioxide capacity was determined, as described in this paper, after saturating at 20° with air containing 5.5 per cent of carbon dioxide. The results are given in Table IX.

The results may be summarized as follows:

1. Blood samples drawn without loss of carbon dioxide (syringe samples) within an interval of a few minutes from three different veins showed within 2.5 per cent the same carbon dioxide contents.

2. The carbon dioxide content of the venous blood from the resting dog (the animal was anesthetized without a struggle) was 2 to 5 per cent greater than that of the arterial blood. Blood

TABLE IX.

(1) Carbon Dioxide Content of Blood from Different Veins Compared with Arterial. (2) Effect of Manner of Drawing Blood on CO_2 Content of Whole Blood and CO_2 Capacity of Plasma.

		Drawn simultaneously.		Drawn simultaneously a few minutes later.		
		Femoral vein.	Jugular vein.	Brachial vein.	Brachial artery.	Femoral artery.
Vol. per cent of CO_2 in whole blood as drawn.	Drawn with syringe (no loss of CO_2)	47.6	46.1	45.1	42.1	43.1
	Blood stream falling through air in receiving flask	43.6	44.1	42.1	41.6	41.1
	Difference	4.0	2.0	3.0	0.5	2.0
Carbon dioxide capacity of plasma from above blood samples.	Plasma from blood drawn with syringe	63.1	60.9	60.9	56.6	58.0
	Plasma from blood drawn into flask	57.1	58.0	58.0	55.1	57.1
	Difference	6.0	2.9	2.9	1.5	0.9

drawn from the brachial vein showed 3 per cent more carbon dioxide than blood drawn simultaneously from the brachial artery.

3. The effect of drawing the blood into an open flask in which the blood stream was allowed to fall through the air for several centimeters was to cause a loss of carbon dioxide from the venous blood such as to bring its CO_2 content to approximately that of arterial. The carbon dioxide held by the venous blood in excess of that of the arterial appears to be given off readily, so that the instant's exposure while the blood was falling into the receiving vessel approximately transformed venous into arterial blood, so far as the carbon dioxide was concerned.

4. The CO_2 capacity of the plasma rose and fell parallel with the CO_2 content of the whole blood from which the plasma was centrifuged. The carbon dioxide bound chemically by plasma saturated at 20° with air containing 5.5 per cent of CO_2 amounted to 15 ±1 volume per cent more than the total carbon dioxide content of the whole blood at the time it was centrifuged.

Within the limits of *this* experiment (compare Experiment VII, *c*), differences in the CO_2 content of the whole blood at the time of centrifugation resulted in approximately equal differences in the carbon dioxide capacity of the plasma, so that the latter remained at a level approximately 15 volume per cent greater than the former. The effect of conditions at the time of centrifugation on the bicarbonate content of the plasma separated is to be attributed to the transfer of electrolytes between plasma and corpuscles under the influence of changing free carbonic acid concentration, discussed on p. 303.

Experiment VII, b. Comparison of Blood Samples Drawn with Syringe and McRae Needle.

The experiment was performed like the preceding, except that for the samples drawn in open vessels a McRae needle was used, so that the conditions entirely simulate those obtained with the use of this apparatus for clinical purposes.

TABLE X.

Carbon Dioxide Contents of Whole Blood Samples from Normal Dog. 1. From Different Blood Vessels. 2. Drawn by: (a) Syringe, without Loss of Carbon Dioxide. (b) McRae Needle in Open Tube.

Vessel.	Vol. per cent of carbon dioxide in blood.			
	Immediately after being drawn.		After standing 6 hours in partly filled tube closed only by cotton plug.	
	Syringe.	McRae needle.	Syringe.	McRae needle.
Femoral vein.....	37.1	33.0	30.4	30.3
Brachial vein.....	35.2	32.4	—	30.8
External jugular vein..	37.1	—	28.9	—
Femoral artery.....	34.0	29.9	26.1	—

The results confirm those of the preceding experiment. In addition they show that whole blood standing in a partly filled tube may lose in 6 hours up to 8 volume per cent of carbon dioxide.

Experiment VII, c. Effect of Different Methods of Drawing Samples on Results Obtained with Venous Blood Heavily Charged with Carbon Dioxide as the Result of Exertion.

The dog used in this experiment exerted himself strenuously against etherization, and consequently the carbon dioxide content of the venous blood was 10.2 volume per cent greater than that of the arterial, instead of the 3 to 5 per cent difference usually noted. The samples were all collected within an interval of a few minutes.

TABLE XI.

		Right jugular vein.	Left jugular vein.	Femoral artery.
		vol. per cent	*vol. per cent*	*vol. per cent*
CO_2 in whole blood as drawn.	Drawn with syringe (no loss of CO_2).....	42.5	42.1	32.0
	Drawn with McRae needle (blood stream falling through air).....	28.6	30.6	25.8
CO_2 bound as bicarbonate by plasma from above blood samples. Plasma is saturated with air containing 5.5 per cent CO_2.	Plasma from blood drawn with syringe.....	52.6	51.6	44.9
	Plasma from blood drawn with McRae needle.....	47.9	46.8	44.0

The results confirm those of the foregoing experiments in showing that allowing the blood to fall for a few centimeters through air when the sample is drawn (with a McRae needle) reduces it to approximately the carbon dioxide content of arterial blood. The fact has additional interest in this case, because the difference between arterial and venous bloods is, presumably on account of the animal's exertions, two or three times as great as that usually observed in resting dogs. Nevertheless the instant's exposure of the falling blood removed all the carbon dioxide in excess of that in the arterial blood, and even somewhat more. The result, however, was, as in the foregoing experiments, to bring the samples drawn with momentary exposure to air closer

to arterial blood in carbon dioxide content than were samples of venous blood drawn without exposure.

Comparison of the carbon dioxide contents of the different whole blood samples and of the carbon dioxide binding powers of the corresponding plasmas affords more examples of the influence of carbonic acid on the acid-base transfer between corpuscles and plasma. Samples of whole blood from which some of the carbon dioxide escaped, during the momentary aeration connected with the use of the McRae needle, yielded plasmas which had also a reduced amount of base capable of binding carbonic acid.

Experiment VII, d. Collection of Blood under Paraffin Oil without Loss of Carbon Dioxide.—The following experiment shows that with centrifuge tube and needle arranged as shown in Fig. 1 one can collect blood samples without appreciable loss of carbon dioxide. The blood in entering the centrifuge tube mixes with the finely powdered oxalate, so that very little additional stirring is necessary in order to prevent clotting. After a sample was drawn the stopper was loosened, and the blood was stirred gently with the inlet tube. Previous experience had shown that if the mixing of the oxalate were attained by more vigorous agitation, such as shaking the tube or turning it upside down repeatedly, a measurable loss of carbon dioxide would occur, even if the layer of oil separating the blood from the air was not broken. Since carbon dioxide is more soluble in the oil than in the water, vigorous agitation of the two fluids results in a partial transfer of carbon dioxide from water to oil. The latter, however, prevents rapid diffusion of the gas away from the surface of the water layer, and if unnecessary agitation is avoided, this form of tube yields results identical with those obtained with syringe samples. The chief advantages over the syringe are in cost, and in the convenience of having the needle on a flexible connection.

The dog used, a female bull terrier of 16 kilos weight, was etherized with the Meltzer-Auer insufflation apparatus. The animal submitted to etherization very quietly; the resting condition is evidenced by the fact that there is only 3 per cent difference in carbon dioxide content between arterial and venous bloods.

It will be noted that the figures in the last column afford another illustration of the effect of CO_2 tension on the acid-base transfer between corpuscles and plasma.

TABLE XII.

Collection of Blood under Paraffin Oil without Loss of Carbon Dioxide.

Blood vessel from which sample was taken.	Instrument used in taking samples.	Total CO_2 content of blood drawn.	CO_2 bound as bicarbonate by plasma saturated at 20° with 5.5 per cent CO_2.
		vol. per cent	*vol. per cent*
Right jugular vein.	Syringe.	33.0	41.3
	Oil tube.	33.0	41.0
	McRae needle.	29.3	32.9
Left jugular vein.	Syringe.	33.9	41.3
	Oil tube.	33.9	41.3
	McRae needle.	33.0	39.6
Femoral artery.	Syringe.	30.3	37.6
	Oil tube.	30.0	37.1
	McRae needle.	25.6	33.3

Experiment VII, e. Effect of Manner of Drawing Blood on Results Obtained from Normal Human Subject.

A sample of blood was drawn from the right arm with a syringe, care being taken to avoid any chance for loss of CO_2. About 1 minute later a sample was taken from the other arm, conditions being the same except that the McRae needle was used in this case. In each case the arm was ligated with a rubber band for about 1 minute before the sample was drawn. The analyses yielded the results shown in Table XIII.

Comparison of analyses *a* and *b* shows, as in the foregoing dog experiments, that use of the McRae needle results in loss of some of the excess carbon dioxide of the venous blood, so that the venous blood is brought closer to arterial (analyses *b*) in its carbon dioxide content.

Comparison of *c* and *d* shows that the approximation of the results to the arterial standard, as a result of the momentary aeration connected with use of the McRae needle, is also noted when the carbon dioxide capacities of the plasmas are considered. The plasma from the venous blood drawn with the McRae needle approximated the plasma from arterialized blood in its carbon dioxide capacity.

TABLE XIII.

Comparison of Blood Samples Drawn from Normal Man with Syringe and McRae Needle.

		Carbon dioxide.
		vol. per cent
a. CO_2 content of whole blood as drawn.	Syringe.	67.7
	McRae needle.	62.1
b. CO_2 content of whole blood arterialized by shaking at 37° with air containing 5.5 per cent of CO_2.	Syringe.	57.2
	McRae needle.	57.2
c. CO_2 bound as bicarbonate by plasma centrifuged from freshly drawn blood and saturated with 5.5 per cent CO_2 at 23°.	Plasma from syringe sample.	76.3
	Plasma from McRae needle sample.	73.3
d. CO_2 bound as bicarbonate by plasma centrifuged from *arterialized* blood (analyses *b* above) and resaturated at 23° with 5.5 per cent CO_2.	Plasma from arterialized syringe sample.	71.0
	Plasma from arterialized McRae needle sample.	71.5

Experiment VIII. Demonstration of Identical Bicarbonate Contents of Venous and Arterial Bloods under Identical Carbon Dioxide Tensions.

A dog of 24 kilos weight was placed under ether with the Meltzer-Auer apparatus and blood samples were drawn as indicated in the following table. Each blood sample was divided into three portions. In one the carbon dioxide content was determined directly (third column). In another the carbon dioxide was determined after saturation at 37.5° of the fresh blood with air containing 5.5 per cent of carbon dioxide. It will be noted that this treatment raised the carbon dioxide of the arterial blood by 10 volume per cent, indicating that the arterial carbon dioxide tension of this animal, presumably because of the artificial ventilation, was considerably less than that of the average man. A third sample of each blood was centrifuged immediately and the plasma bicarbonate CO_2 was determined in the routine way, after saturation of the plasma at room temperature with 5.5 per cent CO_2 in air.

Experiment VII, *d*, is confirmed in showing that blood can be drawn into the "oil tube" without measurable loss of carbon

TABLE XIV.

Blood vessel.	Instrument used in drawing blood.	Total CO_2 content of whole blood as drawn.	Bicarbonate CO_2 of plasma after saturation at 20° with air containing 5.5 per cent CO_2.	Total CO_2 content of whole blood after saturation at 37° with air containing 5.5 per cent CO_2.
		vol. per cent	*vol. per cent*	*vol. per cent*
Left jugular vein.	Syringe.	38.9	48.2	—
		39.3	48.2	—
	Oil tube.	39.3	48.2	46.8
		39.3	48.2	45.9
Left femoral artery.	Oil tube.	36.4	44.5	46.7
		36.4	44.5	45.9

dioxide. The results obtained are identical with those from samples drawn with the syringe.

The figures in the last column show that when arterial and venous bloods are brought to the same content of free carbonic acid, the bicarbonate contents are also equal. From this it follows that blood in passing from arteries to veins in the resting animal does not take up sufficient acid other than carbonic to affect the bicarbonate content appreciably. So far as the content in non-volatile acids is concerned, there is no appreciable difference in the resting animal between blood from the arteries and that from the jugular vein.

The figures in the middle column illustrate again the effect of carbonic acid concentration on the distribution of bases and acids between plasma and corpuscles. Plasma centrifuged from venous blood, with 3 per cent more total carbon dioxide than arterial, showed a bicarbonate CO_2 nearly 4 per cent higher than the arterial plasma.

Experiment IX. Effect of Experimental Acidosis on the Carbon Dioxide Figures and the Hydrogen Ion Concentration of Venous and Arterial Blood.

The animal used was a collie bitch of 14.5 kilos weight, in splendid condition.

At 2.00 p.m. the animal was etherized, and the femoral veins and arteries on both sides were exposed. Ether anesthesia was maintained throughout the experiment by the Meltzer-Auer insufflation method.

At 2.15 blood samples of about 15 cc. each were taken from the right femoral artery and the left femoral vein, the samples being collected under paraffin oil with precautions to prevent loss of carbon dioxide (see p. 306).

At 2.20 the injection of N H_2SO_4 from a burette into the right femoral vein was begun.

At 3.00 50 cc. had been injected. The animal showed marked dyspnea and a pulse of 180. These symptoms disappeared after 10 minutes, and the injection was resumed.

At 3.30 75 cc. of N H_2SO_4 in all had been injected, and the injection was concluded.

At 4.10 a second pair of blood samples was taken, the vessels used being the right femoral artery and the left femoral vein.

At 5 two more samples were taken, in order to ascertain whether the animal's blood indicated that she was overcoming the acidosis.

The hydrogen ion concentrations were determined in a Clark electrode (Clark, 1915), successive portions of the same sample being shaken in the same hydrogen atmosphere until the latter had acquired the carbon dioxide tension of the blood, according to the principle of Hasselbalch's technique (Hasselbalch, 1911, 1913). The figures obtained consequently may be taken as representing the actual hydrogen ion concentration of the blood in the veins and arteries.

The gas analyses of whole blood and plasma were performed as described in this and the succeeding paper.

The results are given in Table XIV. Graphic comparison of the carbon dioxide figures is given in Fig. 6.

TABLE XV.

Effect of Acid Injection on Blood.

	Whole blood as drawn.				Plasma after saturation at 20° with air containing 5.5 per cent CO_2.	
	Arterial.		Venous.			
	Total CO_2.	Reaction.	Total CO_2.	Reaction.	Arterial.	Venous.
	vol. per cent	*pH*	*vol. per cent*	*pH*	*vol. per cent*	*vol. per cent*
Before injection	38.7	7.33	47.9	7.28	49.9	53.2
40 min. after injection of 75 cc. N H_2SO_4	10.1	—	22.1	7.23	17.4	27.7
90 min. after injection	10.1	7.17	22.1	7.17	16.5	26.8

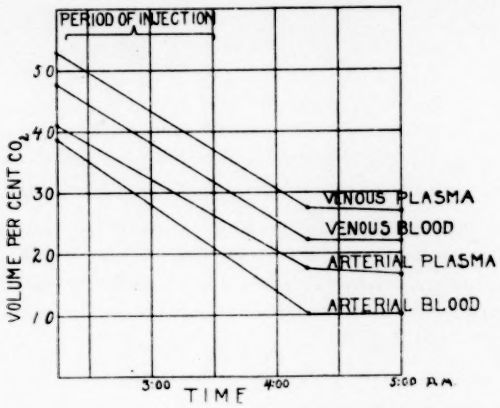

FIG. 6. Effect of acid injection on blood and plasma.

Discussion of the Results of Experiment IX.

1. The curves of Fig. 6 show that in acidosis the arterial carbon dioxide is approximately paralleled in its fall by the other three values determined. For reasons discussed on p. 295 the arterial bicarbonate, or the nearly identical total arterial carbon dioxide, is the ideal figure to determine as a measure of the alkaline reserve of the body. The curves of Fig. 6 indicate, however, that any one of the other three values may also be used as an index of the alkaline reserve, provided the limits of the normal level, and the changes therefrom corresponding to different grades of acidosis, are determined for the value used. The necessity for employing in all clinical and much experimental work one of the values obtained on the venous blood rather than the theoretically preferable arterial figure is, of course, obvious. The value obtained by the technique for acidosis study outlined in this paper, *viz.*, the capacity of the venous plasma to combine with carbon dioxide, has been given the preference in our work chiefly for practical reasons, such as the ease and convenience of making determinations on the plasma as compared with whole blood, the facts that plasma can be preserved for days and even weeks and still show unchanged carbon dioxide binding power when resaturated with 5.5 per cent CO_2, and that unlike the carbon dioxide capacity of the whole blood the carbon dioxide capacity of the

venous plasma maintains its parallelism with the arterial carbon dioxide even in the severe grades of acidosis (Stillman, Van Slyke, Cullen, and Fitz).

2. The greater part of the injected acid did not remain in the blood, but was at once transferred elsewhere and presumably neutralized by the bicarbonate and phosphate reserves in other parts of the body. The amount of blood in a 14 kilo dog may roughly be estimated at 1 liter. The injection of 75 cc. of N acid into this volume of bicarbonate solution would decompose sufficient bicarbonate to reduce its molecular concentration by 0.075. The actual reductions of carbonate noted as the result of the acid injection were the following, the figures being transposed from terms of volume per cent carbon dioxide to molecular concentration:

Arterial whole blood CO_2 content,				reduced	0.0126 M.
Venous	"	"	"	"	0.0115 "
Arterial plasma, CO_2 capacity,				"	0.0104 "
Venous	"	"	"	"	0.0119 "

As bicarbonate furnishes about nineteen-twentieths of the CO_2 of the whole blood, only one-twentieth being free carbonic acid, the whole blood figures as well as those of the plasma (where the CO_2 from H_2CO_3 is subtracted) may be taken as practically bicarbonate figures. The fall, on the average only 0.012 in molecular concentration of both whole blood and plasma, indicates that only about one-sixth of the injected acid remained in the blood, or was neutralized by the blood bicarbonate. The quick transfer between blood and tissues indicates that an acid-base equilibrium is continually maintained between them, even when there is such an enormously rapid influx of acid as occurred in this experiment. There is consequently direct experimental basis for assuming that the bicarbonate concentration of the blood is an index of the alkaline reserve of the entire body.

3. An actual increase in the hydrogen ion concentration of both arterial and venous blood occurred during the experiment. The acidosis was therefore partly uncompensated. Respiration did not lower the free carbonic acid enough to compensate entirely for the lowered bicarbonate, and the failure to do so is evidenced by an increase in the hydrogen ion concentration (fall in

pH) past the extreme normal limits. The failure may, however, be due to the effect of etherization on the respiratory control rather than to the reduction of arterial bicarbonate to one-fourth its previous level. Even severely sick patients appear able to compensate nearly if not quite so severe a grade of acidosis (Peabody, 1914). Michaelis and Davidoff (1912) have found, however, that narcosis of itself causes the respiratory centers to become less responsive to increase in hydrogen ion concentration, and consequently allow free carbonic acid to accumulate until the $\frac{H_2CO_3}{NaHCO_3}$ ratio, and consequently the hydrogen ion concentration, is considerably above normal. Evidence that the etherization was responsible in this experiment is seen in the fact that a further decrease in pH (increase in hydrogen ion concentration) occurred between the second and third bleedings when no acid was injected.

4. The injection of acid apparently results in an increase in the gas exchange occurring with each circulation of the blood. Before the injection the difference in CO_2 content between arterial and venous blood was 9 per cent; after the injection it had increased to 12 per cent. Similar increases in the gas exchange have been noted in other experiments after acid injection. In this case the increase in carbon dioxide occurring as the blood traverses the tissues cannot be attributed to CO_2 set free in the latter by the injected acid, for it was still undiminished $1\frac{1}{2}$ hours after the injection had been finished. The increased gas exchange must be due either to increased rate of oxidation in the tissues, or to decreased rate of blood flow. The problem suggested by this observation, *viz.*, whether acidosis, compensated or uncompensated, produced either experimentally or by disease, results in an increased rate of oxidation, we hope to attack later.

5. The effect of carbon dioxide on the acid-base transfer between corpuscles and plasma is again seen when the bicarbonate CO_2 figures for venous and arterial plasmas are compared. The bicarbonate contents of the sets of plasmas from the two sources differ by nearly the same margins as the total CO_2 contents of the respective whole bloods (see Fig. 6). The results obtained in the experiments of sections VII and VIII show the same relationship between venous and arterial blood and plasma. Con-

sequently the carbonic acid entering the blood from the tissues, during the passage from arteries to veins, must displace nearly an equivalent amount of other acid, which passes into the cells, leaving the plasma at its normal alkalinity. At least part of the acid thus displaced by combined mass action of the carbonic ($H_2CO_3 + NaCl = NaHCO_3 + HCl$) and diffusion is hydrochloric. The results shown in the next experiment indicate that this reaction is the one chiefly concerned when the carbonic acid concentration changes only within the limits likely to occur in the body. The physiological effect of the acid transfer into and out of the cells is to increase tremendously the power of the plasma to take up carbon dioxide without having its hydrogen ion concentration raised above the normal limits. The height to which it is possible to force the plasma bicarbonate by increasing the carbonic acid of the whole blood is indicated by the next experiment.

Experiment X. Effect of Free Carbonic Acid on the Acid-Base Equilibrium between Plasma and Corpuscles.

Blood was drawn with a McRae needle from the arm vein of a healthy man, and the sample was divided into four portions. One was centrifuged at once, and the others were shaken each with 100 volumes of atmosphere containing the proportions of carbon dioxide indicated in Table XVI. Carbon dioxide in the blood plasma was determined in the usual manner, chlorides by the method of McLean and Van Slyke (1915). The experiment reported here is one of a number which yielded similar results. The relationship between chloride and carbonate concentrations is made readily evident by Fig. 7.

For furnishing the blood used in this experiment and performing the chloride determinations we are indebted to our colleague, Dr. Franklin McLean.

The figures for molecular concentration in the plasma of untreated blood (top line of Table XVI) show the relative importance of bicarbonate among the plasma electrolytes. It is, as pointed out by Henderson (1908, *b*), second only to the sodium chloride, the concentration of the bicarbonate being one-fourth to one-third that of the chloride. As shown by Hamburger, the average blood plasma is isotonic with a 0.9 per cent (0.155 molecular) solution of sodium chloride. The average chloride concentration may be taken, as in this blood, at about 0.105 molecular, the bicarbonate

at 0.030 M, the two together therefore at 0.135 M, leaving only about 0.020 M, or one-seventh the total, to be made up of all the other electrolytes in the plasma.

On examining the results from the three blood portions in which the carbonic acid was arbitrarily changed it becomes evident that a part of the resulting changes in plasma bicarbonate are

TABLE XVI.

Effect of Free Carbonic Acid Concentration on Distribution of Chloride and Bicarbonate between Plasma and Corpuscles.

Treatment of whole blood before centrifugation.	Calculated free carbonic acid dissolved in blood as result of treatment.		Bicarbonate of plasma centrifuged from treated blood, then saturated at 20° with 5.5 per cent CO_2.		Plasma chloride.		Changes in plasma concentration caused by treatment of whole blood. Mol. concentration.	
	Vol. per cent CO_2.	Mol. concentration.	Vol. per cent CO_2.	Mol. concentration.	Calculated as gm. NaCl per liter.	Mol. concentration.	Bicarbonate.	Chloride.
No treatment, blood centrifuged as drawn	3.0*	0.0013	69.8	0.0311	6.13	0.1050	—	—
Shaken with 100 volumes of air.	0	0	42.2	0.0188	6.42	0.1098	−0.0123	+0.0048
Shaken with 5.5 per cent CO_2 at 20°, 760 mm	5.3	0.0024	77.9	0.0357	5.95	0.1017	+0.0046	−0.0033
Saturated with pure CO_2 gas at 20°, 760 mm	86.5	0.0386	135.8	0.0607	5.48	0.0939	+0.0296	−0.0111

* Blood as drawn assumed to be saturated with 5.5 per cent CO_2 at 37°, 760 mm.

explainable by the migration of HCl from plasma into corpuscles. Changes within the limits of physiological possibility, such as that caused by changing the carbonic acid CO_2 from 3.0 to 5.3 volume per cent of the blood, may be chiefly accounted for by this shift in hydrochloric acid. When the carbonic acid is greatly altered other electrolytes also become involved in the transfer,

for the changes caused by saturating the whole blood with either pure carbon dioxide or with air practically free of carbon dioxide are, in molecular equivalents, only about one-third as great in the plasma chloride as in the bicarbonate (see last column of Table XV).

In connection with the technique for determining the plasma bicarbonate, these results, showing the extreme possible effects of the acid-base transfer, indicate the magnitude of the changes in plasma which it is possible to cause by varying the carbon

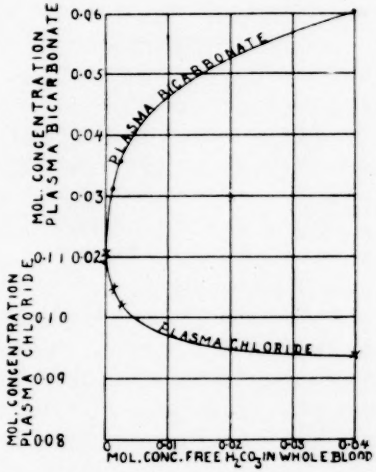

FIG. 7. Effect of free carbonic acid concentration on the distribution of chloride and bicarbonate between corpuscles and plasma. Each square, ordinate or abscissa, represents a change of 0.01 in molecular concentration.

dioxide tension of the whole blood at the time the plasma is separated from the cells. Although these extreme effects greatly exceed those which could be caused by accidental loss or gain of carbon dioxide in handling blood samples, they serve to emphasize the importance of this possible source of error, and the necessity of observing the precautions for handling blood samples given on pages 305 and 306.

Incidentally the data also indicate the effect which carbonic acid changes in whole blood may have on the chloride content

of the plasma. The effect in proportion to the total chloride is only one-third less than the relative effect on the bicarbonate, and if unrecognized could readily become a factor in the results of plasma chloride determinations.

SUMMARY.

Reasons are discussed for basing both the definition of acidosis and the methods for its detection on the blood bicarbonate.

Experiments are detailed showing both *in vivo* and *in vitro* the influence on the plasma bicarbonate of various factors, in particular of the shift of bases and acids between plasma and corpuscles under the influence of changing carbonic acid concentration.

A simple technique has been developed by means of which the capacity of the plasma to combine with carbonic acid under definite tension is determined as a measure of the alkali in excess of acids other than carbonic. The plasma, from oxalated blood, drawn and centrifuged under definite conditions, is shaken at room temperature in a separatory funnel filled with alveolar air from the lungs of the operator, or with an artificial air mixture containing 5.5 per cent of carbon dioxide. The carbon dioxide content of the plasma is then determined by the method described in the next paper. The results are calculated in terms of bicarbonate with the aid of the table on p. 316. The value determined appears to indicate not only the alkaline reserve of the blood, but also that of the entire body.

The results obtained with a given plasma are reproducible to within 1 volume per cent of CO_2, 65 volume per cent being the average normal value for man. In acidosis the carbon dioxide capacity of the plasma falls so far below normal that the method is a most sensitive indicator of this condition and its severity. The simplicity of the technique and the few minutes required for the determination make it available, not only for physiological experiments, but also for clinical routine. Results obtained with normal men and diabetic patients are given in Papers V and VI.

BIBLIOGRAPHY.

Allen, F. M., *Am. J. Med. Sc.*, 1917, cliii, 313.
Austin, J. H., and Jonas, L., *Am. J. Med. Sc.*, 1917, cliii, 81.
Barcroft, J., The Respiratory Function of the Blood, Cambridge, 1914.
Beddard, A. P., Pembrey, M. S., and Spriggs, E. I., *Brit. Med. J.*, 1915, ii, 389. The authors here give references to work done in their laboratories by themselves and by Kennaway and Poulton since 1903.
Benedict, H., *Arch. ges. Physiol.*, 1906, cxv, 106.
Bohr, C., Nagel's Handb. Physiol. Menschen., Braunschweig, 1909, i, 55–220.
Boothby, W. M., *Am. J. Physiol.*, 1915, xxxvii, 383.
Campbell, J. M. H., Douglas, C. C., Haldane, J. S., and Hobson, F. C., *J. Physiol.*, 1913, xlvi, 301.
Christiansen, J., Douglas, C. C., and Haldane, J. S., *J. Physiol.*, 1914, xlviii, 246.
Clark, W. M., *J. Biol. Chem.*, 1915, xxiii, 475.
Fitz, R., and Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 389.
Gettler, A. O., and Baker, W., *J. Biol. Chem.*, 1916, xxv, 211.
Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 29.
Gürber, A., *Maly's Jahresber. Fortschr. Tierchem.*, 1895, xxv, 165–168.
Haldane, J. S., and Priestly, J. G., *J. Physiol.*, 1905, xxxii, 227.
Hamburger, H. J., *Wien. med. Woch.*, 1916, lxvi, 521; *Chem. Abstr.;* 1917, 358.
Hasselbalch, K. A., *Biochem. Z.*, 1911, xxx, 317; *Skand. Arch. Physiol.*, 1912, xxvii, 1; *Biochem. Z.*, 1913, xlix, 451; 1916, *a*, lxxiv, 56; 1916, *b*, lxxvii, 112.
Hasselbalch, K. A., and Gammeltoft, S. A., *Biochem. Z.*, 1915, lxviii, 205.
Hasselbalch, K. A., and Linhardt, J., *Biochem. Z.*, 1916, lxxiv, 17.
Henderson, L. J., *Am. J. Physiol.*, 1905–06, xv, 257; 1908, *a*, xxi, 169; 1908, *b*, xxi, 427; *J. Biol. Chem.*, 1909–10, *a*, vii, 29; *Ergebn. Physiol.*, 1909, *b*, viii, 254; *J. Biol. Chem.*, 1911, ix, 403.
Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1912–13, *a*, xiii, 393; 1913, *b*, xiv, 81; 1914, xvii, 305; 1915, xxi, 37.
Higgins, H. L., *Am. J. Physiol.*, 1914, xxxiv, 114.
Higgins, H. L., and Means, J. H., *J. Pharm. and Exp. Ther.*, 1915, vii, 1.
Howland, J., and Marriott, W. M., *Bull. Johns Hopkins Hosp.*, 1916, xxvii, 63.
von Jaksch, R., *Z. klin. Med.*, 1888, xiii, 350.
Jaquet, quoted by Bohr, p. 109.
Krogh, A., and Krogh, M., *Skand. Arch. Physiol.*, 1910, xxiii, 179.
Lundsgaard, C., *Biochem. Z.*, 1912, xli, 247.
Magnus-Levy, A., *Arch. exp. Path. u. Pharm.*, 1899, xlii, 149.
McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxi, 361.
Michaelis, L., and Davidoff, W., *Biochem. Z.*, 1912, xlvi, 131.
Michaelis, L., and Rona, P., *Biochem. Z.*, 1912, xliv, 232.
Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.

Morawitz, P., and Walker, J. C., *Biochem. Z.*, 1914, lx, 395. This paper includes a partial review of former work on the blood carbon dioxide as a measure of the alkaline reserve.

Naunyn, B., Der Diabetes Mellitus, Vienna, 1906.

Palmer and Henderson, *Arch. Int. Med.*, 1913, xii, 153; 1915, xvi, 109.

Palmer, W. W., *Mass. Med. Soc.*, 1913, xxiv, 133.

Peabody, F. W., *Arch. Int. Med.*, 1914, xiv, 236.

Peters, J. P., *Am. J. Physiol.*, 1917, xliii, 113; *Proc. Soc. Exp. Biol. and Med.*, 1916–17, xiv, 118.

Plesch, J., *Z. exp. Path. u. Ther.*, 1909, vi, 380.

Porges, O., and Leimdörfer, A., *Med. Klin.*, 1915, xi, 219.

Sellards, A. W., *Med. Rec.*, 1914, lxxxv, 126 (editorial).

Sonne, C., *Arch. ges. Physiol.*, 1915–16, clxiii, 75.

Stillman, E., Van Slyke, D. D., Cullen, G. E., and Fitz, R., *J. Biol. Chem.*, 1917, xxx, 405.

Straub, H., *Deutsch. Arch. klin. Med.*, 1913, cix, 223; 1915, cxvii, 307.

Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

Walter, F., *Arch. exp. Path. u. Pharm.*, 1877, vii, 148.

Zuntz, N., Beiträge zur Physiologie des Blutes, Dissertation, Bonn, 1868.

STUDIES OF ACIDOSIS.

II. A METHOD FOR THE DETERMINATION OF CARBON DIOXIDE AND CARBONATES IN SOLUTION.*

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Previous methods for the determination of carbon dioxide in blood have been based chiefly on procedures for either boiling the carbon dioxide out of solution under diminished pressure, or for measuring the gas pressure resulting from addition of acid to the blood in a closed chamber (methods of Haldane and Barcroft).

The chief difficulties encountered in attempting to extract the carbon dioxide from water solution under diminished pressure and measure the gas have been the difficulty of complete extraction and the readiness with which the carbon dioxide is reabsorbed by the water as soon as the vacuum is released. In order to avoid these difficulties recourse has been had to boiling under diminished pressure.[1] The boiling drives out the gas completely from solution, so that it can be collected in a separate chamber and measured over mercury. The process, however, necessitates complicated apparatus, is time-consuming, and is likely to prove troublesome on account of the foaming of such a viscous mixture as blood. This method has, in practice, therefore been completely displaced by the Barcroft-Haldane methods with their relative rapidity and simplicity.[2]

* A preliminary report of the work was published in *Proc. Soc. Exp. Biol. and Med.*, 1914–15, xii, 165. A description of the use of the same apparatus for oxygen determination in blood was reported in *ibid.*, 1917, xiv, 84.

[1] Abderhalden, E., Handb. biochem. Arbeitsmethoden, Berlin, 1910, iii, 678–682.

[2] Barcroft, J., and Haldane, J. S., *J. Physiol.*, 1902, xxviii, 233. Barcroft, J., and Higgins, H. L., *ibid.*, 1911, xlii, 512.

The vacuum extraction principle has advantages, however, as recently demonstrated by Swanson and Hulett, in the short time within which, under low pressure, equilibrium is attained between the gas contents of water and atmosphere, and in the fact that the gases are obtained for direct volumetric measurement.[3]

In their method complete extraction of dissolved gases is not attempted. The solution is shaken at room temperature in an evacuated chamber, where the volume relations between the liquid and the free space are known. A definite proportion of each gas escapes into the free space, and the residue remaining in solution is calculated from the solubility coefficient of the gas. To prevent reabsorption of the gases, Swanson and Hulett transfer them to a burette without releasing the vacuum.

To simplify the application of the same principle to blood analysis and make the entire operation possible with a one-piece apparatus, we have varied the technique by removing the water instead of the gas from the chamber after the extraction, so that the gases can be measured over mercury in a calibrated tube at the top of the same chamber.

Apparatus.[4]

The apparatus as shown in Fig. 1 is designed in proportions especially adapted to determination of carbon dioxide in blood plasma. It consists essentially of a 50 cc. pipette with three-way cocks at top and bottom, and a 1 cc. scale on the upper stem, divided into 0.02 cc. divisions. At the bottom the apparatus is connected by a heavy walled rubber tube with a levelling

[3] Swanson, A. A., and Hulett, G. A., *J. Am. Chem. Soc.*, 1915, xxxvii, 2490. The use of the principle in the development of the present method is due directly to Professor Hulett's suggestion.

[4] The apparatus is manufactured by the Emil Greiner Company, 55 Fulton St., New York.

Caution in Setting up Apparatus.—The jaws of the strong clamp in which the 50 cc. pipette of the apparatus is held should be lined with thick, soft pads of rubber. The pipette has to be clamped firmly, because of the weight of the mercury.

In order to prevent accidental slipping of the apparatus from the clamp, an iron rod of 6 or 8 mm. thickness should be so arranged as to project under cock *f* between *c* and *d*.

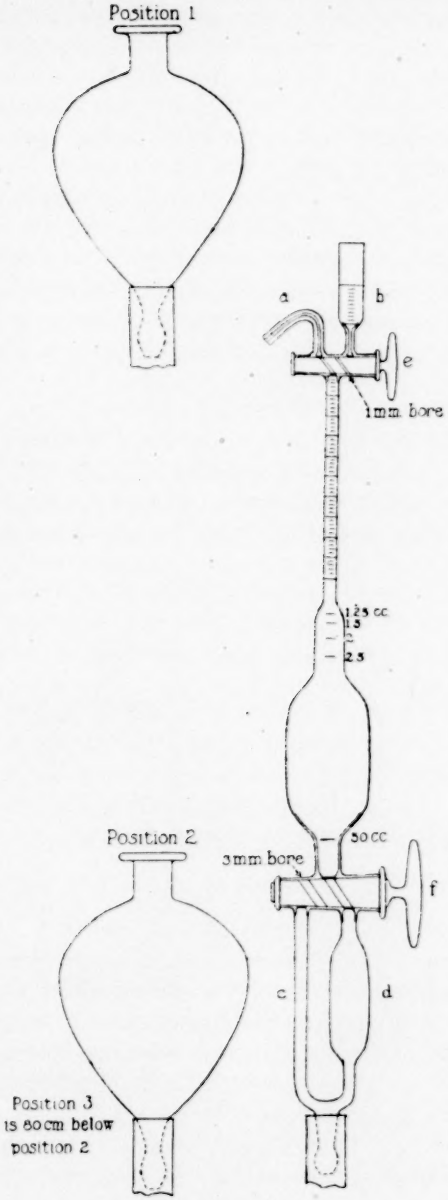
Position 1
a
b
e
1mm bore
1.25 cc
1.3
2.5
Position 2
3mm bore
50 cc
f
c
d
Position 3
is 80 cm below
position 2

FIG. 1.

bulb filled with mercury. The chamber, *d*, serves to draw off the solutions, as above mentioned, after the carbon dioxide has been extracted from them, the other bottom connection, *c*, serving for subsequent release of the vacuum by the entrance of mercury. The apparatus is made of strong glass, in order to stand the weight of mercury without danger of breaking, and is held in a strong screw clamp, the jaws of which are lined with thick pads of rubber. Capillary *a* is used for convenient removal of solutions from the apparatus. It may also for special gas analyses be used to connect the apparatus with an absorption pipette. Three hooks or rings at the levels 1, 2, and 3 serve to hold the levelling bulb at different stages of the analysis.

The calibrated upper stem of the pipette is of such diameter that 1 mm. of length corresponds to about 0.01 cc. By estimating tenths of a 0.02 cc. division, gas volumes can be read to 0.002 cc.

In order to justify such reading the apparatus must be accurately calibrated. To calibrate it one attaches to the outlet at the bottom, a short glass tube drawn out into a capillary tip, and fills the apparatus by suction with distilled water as far as the bottom of cock *e*. By manipulation of cock *f* the water is drawn off, 0.1 cc. at a time, into a weighing bottle, and weighed to within 1 mg.

It is essential that the cocks, especially *f*, shall be held in place so that they cannot be forced out by pressure of the mercury. For this purpose rubber bands may be used, but we have found elastic cords of fine wire spirals applied in the same manner as rubber bands to be stronger and more durable.

The Determination.

Outline.—Briefly stated, the 50 cc. pipette of the apparatus being full of mercury, the solution to be analyzed is acidified within the pipette, the total volume of water admitted being preferably 2.5 cc. A Torricellian vacuum is then obtained in the pipette by lowering the levelling bulb. The carbon dioxide is extracted from the water by a half minute's shaking in the evacuated chamber, and the water is drawn out of the 50 cc. chamber into *d*. Mercury is then readmitted through *c*, and the volume of gas is read at atmospheric pressure in the finely

graduated upper stem of the pipette. The observed volume is corrected by subtraction of the amount (0.04 to 0.05 cc., according to temperature) of air which enters the apparatus dissolved in the water, and by addition of the 4 to 5 per cent of the total CO_2 which remains unextracted because of its solubility in water. These corrections can be determined directly, as will be described, but can be calculated so accurately from the known solubilities of air and carbon dioxide in water that ordinarily only the single reading of the volume of gas first extracted is necessary, the result being calculated by a factor which includes all the corrections.

Testing the Apparatus.—Before a determination is made, the entire apparatus, including the capillaries above the upper cock, is filled with mercury. To test the apparatus for tightness and freedom from gases the mercury bulb is lowered to position 3, so that a Torricellian vacuum is obtained, the mercury falling to about the middle of *d*. The levelling bulb is then raised again. If the apparatus is tight and gas-free the mercury will refill it completely and strike the upper cock with a sharp click. In case there is any gas in the apparatus it serves as a cushion; the click is not heard, and a bubble remains above the mercury. If this is the case, the apparatus must be repeatedly evacuated until the gas has all been removed. Before the apparatus has been used, the rubber tubing, and even the glass walls, hold measurable amounts of gases, which are given off when the apparatus is evacuated. After it has once been freed from these gases, however, it can be used indefinitely without further trouble from this source if no air is admitted again. It is always desirable, nevertheless, before making the first determination of a series, to test the apparatus as above described.

Determination.—The apparatus, including both capillaries above the upper cock, is entirely filled with mercury, and the cup at the top washed free of acid with carbonate-free ammonia (see "Remarks on details" below). The solution to be analyzed is then run from a pipette into the cup. When the solution, like plasma, contains some free carbonic acid as well as carbonate, the tip of the pipette must dip below the surface of the solution in the cup during the transfer. If the liquid were allowed to run through the air in a free stream, carbon dioxide would escape from it. The apparatus is designed to take most conveniently 1 cc. of solution, but satis-

factory determinations can be made with smaller and larger amounts. With the mercury bulb at position 2, and cock *f* in the position shown in the figure, the solution is admitted from the cup into the 50 cc. chamber, leaving just enough above the cock to fill the capillary *b*. The cup is washed twice into the pipette with about 0.5 cc. of water each time, and finally 0.5 cc. of 5 per cent sulfuric acid is run in. In plasma analyses a small drop of caprylic alcohol to prevent foaming should precede the sulfuric acid (see "Remarks on details" below).

It is not necessary that exactly 1 cc. of wash water and 0.5 cc. of acid shall be taken, but the total volume of the water solution introduced must extend exactly to the 2.5 cc. mark on the apparatus, if the special formulas of Table I are to be used. As each portion of water is added, enough is left above the cock to fill the capillary, so that no air can enter the latter with the next solution that is added. After the acid has been admitted a drop of mercury is placed in *b* and allowed to run down the capillary as far as the cock in order to seal the latter. Whatever excess of the sulfuric acid remains in the cup is washed out with a little water.

After all the solutions are in the pipette, the upper cock being closed and sealed with mercury, the mercury bulb is lowered and hung at position 3, and the mercury in the pipette is allowed to run down to the 50 cc. mark, producing a Torricellian vacuum in the apparatus. When the mercury (not the water) meniscus has fallen to the 50 cc. mark, the lower cock is closed, and the pipette is removed from the clamp. Equilibrium of the CO_2 between the 2.5 cc. of water solution and the 47.5 cc. of free space in the apparatus is obtained by turning the pipette upside down fifteen or more times, thus thoroughly agitating its contents. The pipette is then replaced in the clamp.

By turning the lower cock the water solution is now allowed to flow from the pipette completely into *d* without, however, allowing any of the gas to follow it. The levelling bulb is then raised in the left hand, while with the right the cock is turned so as to connect the pipette with *c*. The mercury flowing in from *c* fills the body of the pipette, and as much of the calibrated stem at the top as is not occupied by the gas extracted from the solution. A few hundredths of a cc. of water which could not be

completely drained into *d* float on top of the mercury in the pipette, but the error caused by reabsorption of carbon dioxide into this small volume of water is negligible if the reading is made

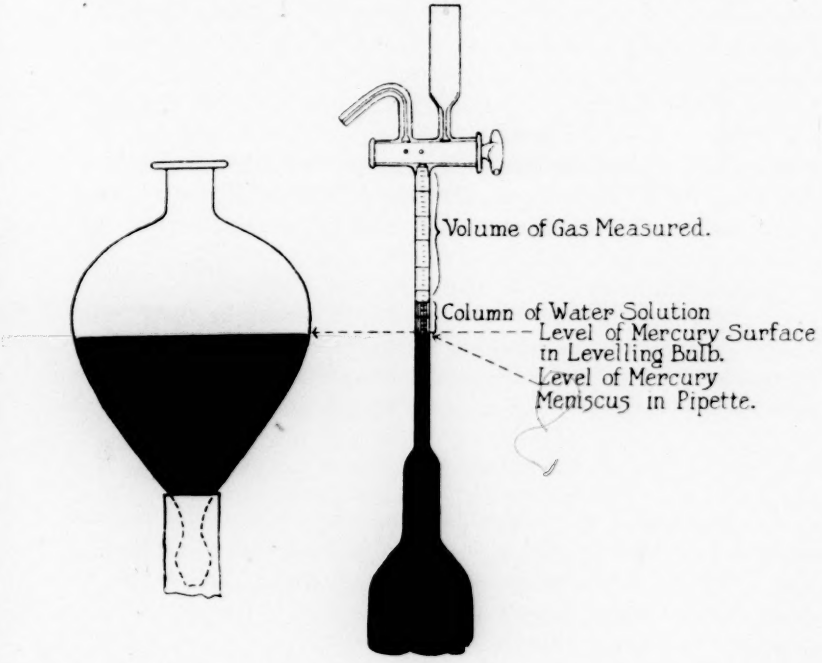
Volume of Gas Measured.
Column of Water Solution
Level of Mercury Surface in Levelling Bulb.
Level of Mercury Meniscus in Pipette.

FIG. 2.

at once. The mercury bulb is placed at such a level that the gas in the pipette is under atmospheric pressure, and the volume of the gas is read on the scale.[5] This concludes the analysis as it

[5] In order to have the column in the pipette exactly balanced by that outside, the surface of the mercury in the levelling bulb should be raised until it is level with the mercury meniscus in the pipette, and then, for entire accuracy, raised above the latter meniscus by a distance equal to $\frac{1}{14}$ the height of the column of water above the mercury in the pipette (Fig. 2). As the water column is, as a rule, only about 10 mm. high, the correction that has to be estimated in less than 1 mm. of mercury, *i.e.*, the entire correction for the water column, is not enough to influence results appreciably.

is ordinarily done, and the results are calculated with Table I, or, when plasma CO_2 capacity is being determined, with the table on p. 316 of the preceding paper.

After the determination has been finished, the levelling bulb is again lowered without opening the upper cock, and most of the mercury is withdrawn from the pipette through *c*. The water solution from *d* is readmitted and, the levelling bulb being raised to position 1, the water solution, with a little mercury, is forced out of the apparatus through *a*.[6]

The apparatus is now ready for another determination. It is not necessary to wash it out, since the few drops of water which remain in it attached to the walls hold no measurable amount of carbon dioxide. One can, consequently, perform series of determinations at the rate of one every 3 or 4 minutes.

When not in use the entire apparatus should be filled with water. Aside from keeping the cocks properly greased, this is about the only special attention it requires. The mercury is occasionally cleaned by straining it through chamois skin.

Remarks on Details of the Determination.

As a precaution it is advisable immediately before starting the analysis to wash the cup of *b* with a carbonate-free solution of about 1 per cent concentration of ammonia. This treatment assures an alkaline reaction in the cup, which is essential to prevent the possible escape of carbon dioxide from the solution analyzed. The precaution is particularly advisable because the last fluid regularly passing through *b* in each determination is 5 per cent sulfuric acid. Ordinary ammonia solution can be made carbonate-free by adding a small amount of saturated barium hydrate solution. The barium carbonate is filtered off, and the excess of barium remaining is precipitated with a little ammonium sulfate.

An ordinary 0.5 or 1.0 cc. Ostwald pipette may usually be employed in transferring the solution to be analyzed to the receiving

[6] It is well to have a funnel draining into a special vessel to catch the water residues and mercury overflow from *a*. A considerable amount of mercury is thus regained if many analyses are run. It requires only straining through cloth or chamois skin to prepare it for use again.

cup *b*. The pipettes may be calibrated either for delivery to a mark on the lower stem, or for blowout delivery. In the latter case the final drop is expressed by closing the top of the pipette with the forefinger of one hand, and warming the bulb with the palm of the other. The expansion of the air in the bulb of the pipette forces the drop out at the tip, without following it with a stream of air. The blowout pipette may be used for any solution in which the CO_2 tension is less than one-fifth of an atmosphere. If it is higher, the pipette calibrated to deliver between two marks must be used, as an appreciable amount of CO_2 escapes from the upper layer of solution in the pipette. In the doubly marked instrument this layer is not expelled, and does not influence the analysis.

If the solution analyzed is, like blood plasma, viscous and likely to foam when the gas leaves it, it is convenient, though not absolutely necessary, to add a small drop of caprylic alcohol. With plasma, 0.02 cc. of the alcohol is sufficient to prevent foaming, and does not affect the results. It is measured most conveniently from a burette made by fusing a capillary stop-cock onto a pipette graduated into 0.01 cc. divisions. The drop of caprylic alcohol is placed in *b* before the 0.5 cc. of acid is added, and is permitted to flow entirely into the capillary above *e*. It is then pushed into the pipette ahead of the acid when the latter is admitted. If the alcohol were not trapped in the capillary, it would float on top of the acidified water solution next added and be impossible to transfer to the chamber below.

It is desirable to keep the amount of caprylic alcohol small, as larger amounts may appreciably increase the results, because of the vapor tension of impurities which the alcohol may contain, and because it dissolves much more air per unit volume than does water. Every lot of caprylic alcohol used should be tested by analyzing standard carbonate solutions with and without the addition of the alcohol. If the latter causes an appreciable error it should be redistilled, preferably under reduced pressure. We have always found Kahlbaum's caprylic alcohol, "*Oktylalkohol, Sekundär I*" to give satisfactory results without purification, but this is not the case with all brands. Satisfactory caprylic alcohol can be made by letting castor oil stand over night with an equal volume of concentrated sodium hydrate solution, and distilling from an oil bath (Beilstein, i, 238).

Direct Determination of the Dissolved Air and of the Carbon Dioxide Not Removed by the First Extraction.

The gas obtained by a single extraction represents, according to the temperature, from 95 to 96 per cent of the carbon dioxide that was in the solution analyzed, plus all of the air, 0.04 to 0.05 cc., dissolved under atmospheric pressure by the 2.5 cc. of water introduced into the pipette. This volume of air, which must be deducted from the total gas volume, we usually calculate from the solubility of air in water at the room temperature prevalent (the solutions being shaken with air before use, in order to make certain that they are saturated). The air can, however, readily be determined after absorption of the carbon dioxide. For the direct determination, one admits through the upper cock, after measuring the gas, a little 10 per cent potassium hydrate solution, which in running down the inner wall of the calibrated tube absorbs all the carbon dioxide. The mercury bulb is then held at the proper level[5] and the volume of residual air is read off on the upper part of the scale.

As stated above, from 4 to 5 per cent of the carbon dioxide remains in solution in the water after equilibrium has been reached. This is because the Henry coefficient $\frac{CO_2 \text{ per cc. atmosphere}}{CO_2 \text{ per cc. water}}$ varies between 1 and 0.8 over the ordinary range of room temperature. In order to determine this unextracted carbon dioxide directly, the gas and water in the top of the pipette are, after the gas volume is read, forced out through *a*. In case alkali has been run in for a direct determination of the air, a little acid is run in to wash out the pipette, and is then removed through *a*. The pipette is then evacuated, the mercury run down to the 50 cc. mark, and the water solution in *d* readmitted into the 50 cc. chamber. The apparatus is then shaken as before by inverting it ten or twelve times, the water solution is drawn off into *d*, and the extracted gas measured in the calibrated upper stem of the pipette. The second extraction removes 95 per cent of the 4 or 5 per cent of the total carbon dioxide which was not taken out by the first extraction. Consequently the two extractions give 99.8 per cent of the carbon dioxide that was dissolved in the solution analyzed.[7]

[7] No measurable amount of air is obtained by the second extraction. The solubility of air is so slight that it is all removed by the first extraction.

Calculation of Results.

When the determination is direct throughout, the carbon dioxide being completely obtained by two extractions, while the admixed air in the first gas extract is measured after absorption of the carbon dioxide, one has merely to multiply the total volume of CO_2 obtained by the weight of 1 cc. of moist CO_2 at the prevailing temperature and barometric pressure.

The second extraction can, however, like the determination of admixed air in the first gaseous extract, be dispensed with by utilizing Henry's law. The volume of water and of the free space in the evacuated pipette being fixed, the proportion of carbon dioxide remaining in the water when equilibrium has been established can be accurately calculated from the solubility coefficient in a manner which will be discussed below. By utilization of this principle the entire determination can be reduced to one extraction and the measurement of the gas extracted, the correction for unextracted carbon dioxide, as well as that for the air carried into the system in water solution, being made by calculation, with results as accurate as can be obtained when both corrections are determined by direct measurement. In fact, the calculation of these two corrections by Henry's law is as a rule somewhat more accurate than their direct determination, as the solubility coefficients for air and carbon dioxide are accurately known, and the experimental error involved in the two extra readings is dispensed with when the corrections are calculated from the solubilities.

The formula for the calculation is developed as follows.

$V_{0°, 760}$ = Volume of carbon dioxide, reduced to 0°, 760 mm. in solution analyzed.

V = Volume of gas, obtained by one extraction and measured at atmospheric conditions of t° temperature and B mm. barometric pressure.

f = temperature factor for reduction of volume of gas, measured moist at t°, to volume occupied by dry gas at 0°.

S = cc. of water solution introduced into apparatus.

α_{air} = solubility coefficient of air in water.

α_{CO_2} = " " " CO_2." "

The solubility coefficients α_{air} and α_{CO_2} used here express the cc. of air or CO_2 measured at t° (not 0°) dissolved by 1 cc. of water in contact with pure air or CO_2.

Since S cc. of water, saturated with air at atmospheric pressure and therefore containing $S\,\alpha_{air}$ cc. of air, is introduced into the apparatus, and is completely removed by the first extraction, the volume of CO_2 in the CO_2 + air mixture measured after a single extraction is $V - S\,\alpha_{air}$.

Since, however, $\frac{S}{50}$ of the vacuum chamber is occupied by water at the time of the extraction, $\frac{S}{50}\,\alpha_{CO_2}$ parts of the carbon dioxide present remain dissolved in the water phase. The volume of CO_2 extracted is therefore only $1 - \frac{S}{50}\,\alpha_{CO_2}$ of the total, and must be divided by this value to give the total.

Therefore:

$$\textit{Total } CO_2 \textit{ measured at room temperature} = \frac{V - S\,\alpha_{air}}{1 - \frac{S}{50}\,\alpha_{CO_2}}$$

When, as in the routine analyses, S = 2.5 cc. the equation becomes

$$\textit{Total } CO_2 \textit{ measured at room temperature} = \frac{V - 2.5\,\alpha_{air}}{1 - 0.05\,\alpha_{CO_2}}$$

Introducing the corrections for temperature and barometer, we have

$$(1)\quad V_{0°,\,760} = (V - 2.5\,\alpha_{air}) \times \frac{f}{1 - 0.05\,\alpha_{CO_2}} \times \frac{B}{760}$$

The values of f, α_{CO_2}, and α_{air} with temperature coefficients sufficiently exact to affect results calculated with the above formula by not more than 0.1 per cent between 15° and 30° are[8]

$$f = 0.999 - 0.0046\ t$$

$$\alpha_{CO_2} = 1.412 - 0.0225\ t$$

$$\alpha_{air} = 0.0255 - 0.00033\ t$$

Introducing these values, we have

$$V - 2.5\,\alpha_{air} = V - 0.063 + 0.0008\ t$$

$$\frac{f}{1 - 0.05\,\alpha_{CO_2}} = 1.074 + 0.0061\ t + 0.000{,}005{,}5\ t^2$$

$$= 1.074 + 0.0059\ t \text{ (between 15° and 30°)}$$

---

[8] The values for α_{CO_2} and α_{air} are those of Bohr and Bock (*Chem. Kalendar*, 1912, i, 271 and 275). They are recalculated so that the values are here expressed in volumes of CO_2 at t°, 760 mm., instead of 0°, 760 mm., as in the original.

Whence:

$$(2)\quad V_{0°,\,760} = (V - 0.063 + 0.0008\,t)\,(1.074 - 0.0059\,t)\,\frac{B}{760}$$

To express the results in mg. of carbon dioxide, we multiply the second factor by 1.964, the weight of 1 cc. of the gas at 0°, 760. mm.

We then have:

$$(3)\quad \text{Mg. } CO_2 = (V - 0.063 + 0.0008\,t)\,(2.109 - 0.0116\,t)\,\frac{B}{760}$$

For convenience in calculating results obtained at different temperatures the following table is given. If, for example, 0.69 cc. of gas are measured at 22°, 750 mm., the mg. of CO_2 indicated $= (0.690 - 0.045) \times 1.854 \times \frac{750}{760}$ $= 1.180$ mg.

If the result is desired in cc. of CO_2 reduced to 0°, 760, it is $(0.690 - 0.045) \times 0.944 \times \frac{750}{760} = 0.601$ cc.

The degree of accuracy attainable by this method is indicated by the following analyses. The solutions were made by weight from Merck's reagent anhydrous Na_2CO_3, which had been heated in an oven to insure its dryness. 1 cc. of solution was used for each analysis. The second extraction of carbon dioxide was performed in each case, and the volume of carbon dioxide obtained by it is given in the last column. The next to the last column gives the volume of carbon dioxide allowed for by Equation 1 as the residual volume of CO_2 gas not removed from solution by the first extraction; *i.e.*, the difference between the values calculated by Equation 1 as it stands, and the values calculated without the term $-0.05\,\alpha_{CO_2}$ in the denominator. Comparison of the last two columns shows that the calculated and observed amounts agree within the limit of error of the latter.

The results show both that the method is free from serious error, and that the extra labor of determining the "dissolved air" to be subtracted, and the "unextracted carbon dioxide" to be added to the first reading, can be dispensed with; as these corrections can be calculated with probably even greater accuracy than they can be determined in this apparatus. Consequently the necessary labor is reduced to extracting the carbon dioxide once from its solution, reading the volume of gas obtained, and calculating the result with the aid of Table I. The results of blood plasma analyses are given in accompanying papers.

TABLE I.

Carbon Dioxide Indicated by Reading of V Cc. of Gas after a Single Extraction.

Temperature of analysis.	Air dissolved in 2.5 cc. H_2O.* Subtract this from V and multiply result by A to calculate mg. CO_2, by C to calculate cc. CO_2 reduced to 0°, 760 mm.	A.	C.
°C.	cc.	mg.	cc.
15	0.051	$\frac{B^{**}}{760} \times 1.935$	$\frac{B^{**}}{760} \times 0.985$
16	0.050	" 1.924	" 0.980
17	0.049	" 1.912	" 0.974
18	0.048	" 1.900	" 0.968
19	0.048	" 1.889	" 0.962
20	0.047	" 1.877	" 0.956
21	0.046	" 1.866	" 0.950
22	0.045	" 1.854	" 0.944
23	0.045	" 1.842	" 0.938
24	0.044	" 1.831	" 9.932
25	0.043	" 1.819	" 0.927
26	0.042	" 1.808	" 0.921
27	0.041	" 1.796	" 0.915
28	0.040	" 1.784	" 0.909
29	0.040	" 1.773	" 0.903
30	0.039	" 1.761	" 0.897

* This correction can be used when, as in the case of plasma and most other solutions, the oxygen and nitrogen dissolved by water from air at atmospheric pressure are the only gases besides carbon dioxide given off in appreciable amounts by the acidified solution. In the analysis of whole blood, however, one also extracts part of the oxygen bound by the hemoglobin, and in this case the volume of gases other than CO_2 must be determined directly after absorbing the CO_2 with 10 per cent KOH solution.

** For convenience the values of $\frac{B}{760}\left(=\frac{\text{barometric pressure in mm.}}{760}\right)$ for ordinary atmospheric pressures are given after Table I at the end of the paper immediately preceding this.

TABLE II.

Analyses of Standard Carbonate Solutions.

Na_2CO_3 per liter solution.	Volume of gas observed in analysis of 1 cc. solution. First extraction.	Temperature.	Barometer.	CO_2 per cc. solution. Calculated by Table I.	CO_2 per cc. solution present.	Unextracted CO_2.	
						Allowed for in calculation.	Obtained by second extraction.
gm.	*cc.*	*°C.*	*mm.*	*mg.*	*mg.*	*cc.*	*cc.*
3.616	0.862	24	758	1.491	1.500	0.037	0.035
"	0.866	24.5	758	1.501	"	0.036	0.033
"	0.868	25	758	1.504	"	0.036	0.035
"	0.867	25	758	1.502	"	0.036	0.034
2.410	0.595	24	756	1.004	1.000	0.024	0.022
"	0.597	24.5	756	1.006	"	0.024	0.024
"	0.592	23.5	756	1.000	"	0.024	0.024
"	0.594	23.5	756	1.003	"	0.024	0.026
1.205	0.318	22	756	0.501	0.500	0.012	0.010–0.015*
"	0.318	22	756	0.501	"	0.012	"
"	0.316	22	756	0.498	"	0.012	"
"	0.315	22	756	0.497	"	0.012	"

* Entirely accurate measurement was impossible so near the zero point of the scale.

Analysis of Solutions, Such as Whole Blood, Which Yield Other Gases, in Addition to Carbon Dioxide and the Amount of Air Dissolved by Water at Atmospheric Pressure.

With such solutions the carbon dioxide must be determined directly by absorption with potassium hydrate solution, as described on p. 356. The carbon dioxide volume thus obtained is multiplied by the value of A or C in Table I corresponding to the temperature of the analysis.

This method must be utilized when the carbon dioxide content of whole blood is determined, as about two-thirds of the oxygen combined with the hemoglobin is obtained by the extraction of the acid solution.

Micro-Apparatus for Estimation of Carbon Dioxide in Small Volumes of Solution.

The micro-apparatus described below is designed to measure within 1 volume per cent the small amounts of carbon dioxide

(0.1 to 0.15 cc.) in 0.2 cc. of blood plasma. As will be seen, the principle of extraction in a Torricellian vacuum is utilized, as with the larger apparatus, but the manner of application is somewhat different from that followed with the latter. Instead of measuring the gas in the extraction chamber after removal of the water, the gas is transferred to a separate measuring chamber without release of the vacuum.

Apparatus.—The nature of the apparatus is evident from the figure. The use of a glass tube instead of rubber as part of the connection between the apparatus and the levelling bulb is necessary because the minute amounts of gas which rubber may give off are sufficient to affect results appreciably. The curve in the lower part of this glass tube serves as a trap to catch any small gas bubbles which might come from the rubber tube forming the remainder of the connection with the levelling bulb. The reasons for other differences in structure become evident when the directions below for using the apparatus are read.

The capillary tube in which the gases are measured is divided into 100 divisions of 0.002 cc. each, numbered from 1 to 100 rather than in absolute volumes, so that when 0.2 cc. of carbonate solution is analyzed each division indicates a volume equal to 1 per cent that of the solution. The different parts of the micro-apparatus are in the same relative proportions as the corresponding parts of the larger apparatus, being one-fifth as large. Consequently when 0.5 cc. of total water solution is introduced the same formula (Table I) can be used for calculating results as in the case of the large apparatus, each division of 0.002 cc. on the smaller corresponding to 0.01 cc. on the larger.

Determination.—The apparatus being entirely filled with mercury, the solution or plasma, usually 0.2 cc., is measured into cup *a* at the top of the apparatus, the tip of the pipette being kept in contact with the liquid in the cup during the delivery. An Ostwald pipette with a heavy walled capillary stem of about 1 mm. inner diameter is used. The pipette is calibrated to deliver 0.2 cc. between two marks, the lower of which is 3 to 4 cm. above the tip, as with such small amounts more accurate results are obtained by draining the pipette between two points than by blowout delivery. The solution is washed from *a* into *b* with two portions of about 0.1 cc. of water each, the water being dis-

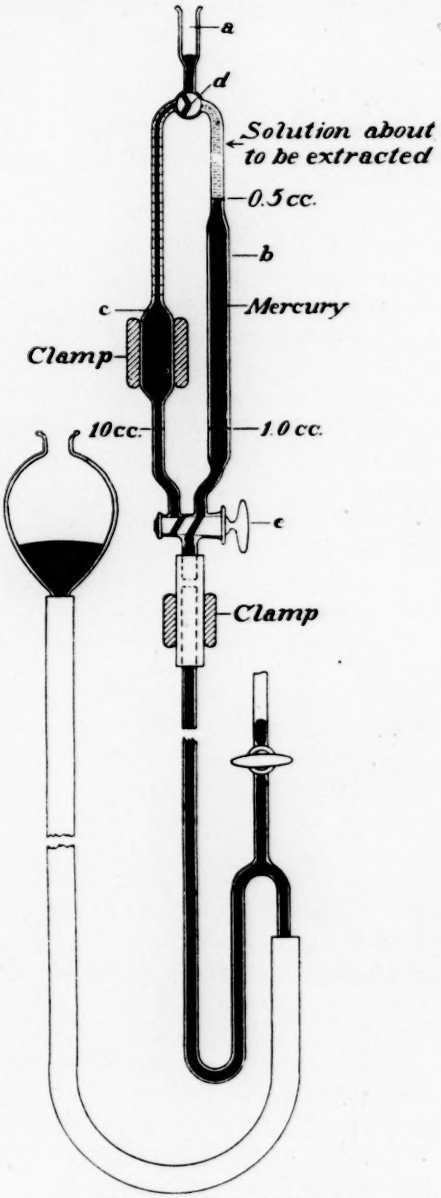

FIG. 3. Micro-apparatus.

tributed about the lower part of the wall of *a* with a fine-pointed medicine dropper. Enough 5 per cent sulfuric acid is then admitted to fill *b* down to the 0.5 cc. mark. No caprylic alcohol need be added, even with plasma, to prevent foaming. The admission of the successive portions of liquid from *a* into *b* is best controlled by leaving open the connection between *a* and *b*, and governing the inflow with cock *e*.

The 0.5 cc. of water solution being all within *b*, cock *d* is turned to connect *a* with *c*, and a little mercury is forced up into *a* where it serves to provide the necessary mercury seal to cock *d*.

Cock *d* is now turned to the position shown in the drawing, which represents the apparatus at this stage of the determination. Chamber *b* is evacuated till the mercury has fallen to the 10 cc. mark. Cock *e* is then closed, the clamp about *c* is loosened, and the apparatus is shaken by moving it to the horizontal position and back a dozen times. Extraction seems even quicker than with the larger apparatus. The clamp below *e* is not loosened during this or any other part of the analysis.

The apparatus is returned to the upright position, and *c*, which has hitherto been full of mercury, is evacuated. Then, the levelling bulb being raised to about the level of *e*, *d* is turned to connect *b* and *c*, and mercury is at once admitted through *e* into *b*. As *b* fills with mercury the rarefied gases in it pass over into *c*. When the solution in *b* has risen to the narrowed upper portion of the chamber the rate of flow is retarded, and is cautiously allowed to progress until the meniscus of the water just or almost reaches cock *d*. Cock *e* is then closed and *d* is turned to connect *a* and *b*. Mercury from *a* flows into the evacuated bore of *d* and seals the cock with a completeness which is necessary for an accurate final reading of the gas now in *c*. The amount of gas trapped in the bore of *d* is negligible.

Cock *e* is now turned to admit mercury into *c*, and the volume of gas trapped in the calibrated capillary at the top of *c* is read off at atmospheric pressure, the levelling bulb being so held that the surface of the mercury in it is level with the mercury meniscus in the capillary. The results are calculated by the same tables used for the larger apparatus. The barometric pressure must be corrected, however, for the effect of capillary attraction on the mercury in the calibrated capillary. This correction is read-

ily determined by connecting *c* with the outer atmosphere through *d*, holding the levelling bulb near to the calibrated capillary, and measuring the difference between the levels of the mercury surfaces in the bulb and the capillary respectively. In our apparatus, with a capillary of somewhat more than 1 mm. bore, the capillary action held the mercury meniscus 4 mm. below the mercury surface in the levelling bulb. Consequently 4 mm. is deducted from barometric readings in making the gas calculations.

Before every analysis, even of a series made at one time, the apparatus must be cleared of any minute bubbles by lowering the mercury bulb and evacuating both chambers *b* and *c* as well as the tube for some distance below cock *e*. When the mercury is readmitted a little is forced up into *a* from both chambers, carrying with it any bubbles which may have been detached from the walls. This precaution is absolutely necessary if results are to be obtained within the 1 per cent limit of accuracy.

Air is admitted into the apparatus as seldom as possible because it is adsorbed by the glass walls and held, even after readmission of mercury, in amounts sufficient to cause gross errors. In case air is admitted, it must be removed by evacuating the apparatus twice in the manner described in the preceding paragraph before an analysis is performed.

Calibration of the Micro-Apparatus.

The capillary measuring tube may be calibrated in two ways, either by weighing the mercury which it delivers, or by analyzing standard solutions of Na_2CO_3 and ascertaining the difference between the observed volumes and those calculated with the aid of Table I.

In the mercury calibration it is essential that the capillary should be *wet*, because, from condensed water vapor, it is always wet when analyses are performed. Consequently a drop of water is run down the calibrated capillary and then expelled by mercury. If the tube is clean, just enough water will remain attached to the walls to form a layer over the mercury so thin that the menisci of mercury and water meet in the middle of the capillary. This is the condition under which readings of analyses are made.

TABLE III.

Na_2CO_3 per liter of solution.	Temperature.	Barometer.	Gas reading observed. Scale divisions of 0.002 cc.	Gas reading calculated. Scale divisions.	Scale correction.	Average correction.
gm.	°C.	*mm.*				
3.616	23	748	86.0	87.1	+1.1	
	"	"	85.9	"	+1.2	+1.2
	"	"	85.9	"	+1.2	
	"	"	85.9	"	+1.2	
1.446	"	"	38.6	37.8	−0.8	
	"	"	38.3	"	−0.5	−0.6
	"	"	38.3	"	−0.5	
0.723	"	"	22.1	21.0	−1.1	
	"	"	22.0	"	−1.0	−1.1
	"	"	22.2	"	−1.2	
	"	"	22.1	"	−1.1	

For delivery of the mercury, a glass tube drawn out into a very fine capillary is attached to the tube below cock *e*. The delivery of the mercury is controlled with cock *e*, and the tip of the capillary is touched to the mercury in the weighing bottle after the delivery of each portion.

For calibration with standard carbonate solutions one simply makes analyses in the usual manner.

Fig. 4 indicates the error that would be introduced by calibrating the apparatus with dry walls; it also shows how closely the results of calibrating by means of standard carbonate agree with those by the mercury method when the mercury is delivered from the moist capillary.

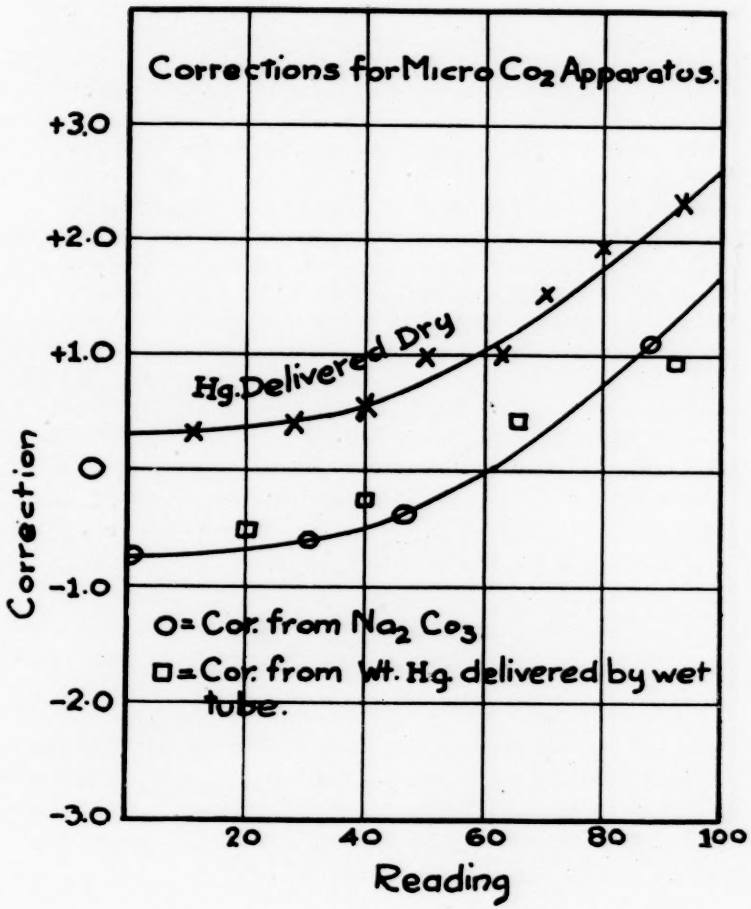
Corrections for Micro Co_2 Apparatus.
+3.0
+2.0
+1.0
0
-1.0
-2.0
-3.0
Correction
Hg. Delivered Dry
O = Cor. from $Na_2 Co_3$
□ = Cor. from wt. Hg. delivered by wet tube.
20
40
60
80
100
Reading

FIG. 4.

SUMMARY.

A simple one piece apparatus is described for determination of the carbon dioxide or carbonate content of water solutions. It has been designed especially for analysis of 1 cc. samples of blood plasma, but is applicable to water solutions in general as well as to the determination of dissolved gases other than carbon dioxide.

The entire analysis is performed at room temperature, requires about 3 minutes, and without especial precautions is capable of accuracy to within 1 per cent of the amount determined.

A micro-apparatus designed on a similar principle is described. With it the carbon dioxide content of 0.2 cc. of plasma can be determined with an accuracy of 1 volume per cent.

STUDIES OF ACIDOSIS.

III. THE ELECTROMETRIC TITRATION OF PLASMA AS A MEASURE OF ITS ALKALINE RESERVE.

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It has been definitely established that the reaction of the blood is, under normal conditions, constant (Lundsgaard, 1912) and may be kept constant even under pathological conditions involving great lowering of the alkaline reserve (Michaelis, p. 105; Benedict, 1906; Peabody, 1914; for more detailed discussion see Paper I of this series). Consequently, the hydrogen ion concentration (C_H) of the blood as drawn from the veins cannot be taken as an indicator of the alkaline reserve.

We may expect, however, that when a given amount of acid is added to the blood, the resultant change in C_H will be greater the less the reserve alkali; or conversely, that less acid will be necessary to produce a given increase in C_H. The latter principle is essentially that used in methods for titration of the blood with indicators, acid being added until the C_H is reached at which the indicator changes color; and titration methods have contributed greatly to our knowledge concerning the alkaline reserve of the blood and its changes in acidosis (Jaksch, Magnus-Levy, Pembrey, and Spriggs). The interpretation of such results, however, is clouded by two deficiencies inherent in the method. First, the indicators (Bjerrum, 1915), such as methyl orange, which can be used to titrate carbonates as alkali change color at such a high C_H that the titration measures, in addition to the bicarbonate, also an acid binding power of the proteins quite out of proportion to the amounts of acid which these substances can bind within the limits of C_H possible in life (the phosphates of whole blood also act as buffers like proteins, but they play a minimal rôle in plasma). Second, the proteins dimin-

ish by their buffer effect and by absorption of indicator the sharpness of the end-points, and thereby decrease the accuracy of the titrations. Attempts to overcome these difficulties by precipitating the proteins with neutral agents such as ammonium sulfate are criticized because the precipitated protein carries down with it a considerable part of the alkali (Hoppe-Seyler). Of the above two difficulties the first is inherent in every mode of titrating blood against strong acids. The second, however, can be avoided by determining the C_H of the end-point electrometrically instead of by indicators, so that even with small amounts of blood or plasma results reproducible with a high degree of accuracy can be obtained. The present paper presents the results of an attempt to develop the electrometric titration into a form practicable for use with normal and pathological plasmas,—and the comparison of the method so developed with the determination of the carbon dioxide combining capacity of the plasma (Van Slyke and Cullen, 1917), and of the carbon dioxide tension of the alveolar air (see Paper VI of this series).

The principles of the gas chain method and its practice have been so thoroughly reviewed in recent literature (Soerensen, 1912; Hildebrand, 1913; Michaelis, 1914; Clark and Lubs, 1916) that only the necessary details are given here.

Apparatus.

All determinations of the H^+ concentration were made by use of the well known gas chain.

$$\mathrm{Hg} \;\big|\; \mathrm{HgCl} \;\big|\; \text{N/10 KCl} \;\big|\; \begin{matrix}\text{Saturated}\\ \text{KCl}\end{matrix} \;\big|\; \text{Solution} \;\big|\; \mathrm{H_2} \;\big|\; \mathrm{Pt}$$

The mercury for the calomel cells was purified in the wet way, distilled three times by Hulett's method (1911), and filtered. The calomel was made from this mercury by the method of Loomis and Acree (1911).[1] The N/10 KCl solution was prepared by weight from recrystallized Kahlbaum's KCl and the standardi-

[1] Electrodes prepared from this calomel have not been very satisfactory. In later work with the gas chain the calomel has been prepared electrolytically as described by Ellis (1916). The calomel has been kept under acid and prepared for use by washing by decantation only. Cells prepared from such calomel remain entirely constant for long periods.

zation verified by chloride determinations by the McLean and Van Slyke method.

The hydrogen was taken from tanks of the Standard Oxygen Company and was washed through solutions of $HgCl_2$, $KMnO_4$, pyrogallol (twice), dilute H_2SO_4, and water. This proved to be a convenient and satisfactory source of hydrogen.

The electrodes were made from No. 16 and No. 18 platinum wire sealed into glass tubes and platinized with platinum black. Small Clark electrode vessels of about 2 cc. capacity were used, together with the ingenious Clark shaking device (Clark, 1915). This vessel is similar in principle to that of Hasselbalch (1911, 1913), but is superior to it both practically and theoretically. It is designed to give maximum surface of solution and elimination of dead space, and to reduce the contact potential between the solution and saturated KCl solution to a negligible quantity. It is easily manipulated, requires only a small amount of fluid, and gives such thorough contact of solution and electrode that equilibrium is established inside of a few minutes. This apparatus is especially convenient when working with carbon dioxide containing fluids, for it allows easy and economical renewal of the solution without change of hydrogen, the procedure proposed by Hasselbalch. The substitution of a 60° 3-way stop-cock with a 2-way key for the lower stop-cock of the Clark vessel is a decided convenience.

Several calomel electrodes were kept on hand, and compared frequently with one another. An absolute standard was thus maintained, and the readings corrected with it. Weston cells calibrated by the Bureau of Standards gave the standard potential. The readings were taken during the earlier part of the work with a bridge of 1,110 ohms resistance boxes and electrometer as zero point instrument. The external resistance was so adjusted that ohms equalled millivolts. Later a Leeds and Northrup potentiometer with a galvanometer as zero point instrument was installed.

The determinations were run at room temperature, 18–24°, and temperature corrections applied. The accuracy of the entire determination was tested often by means of Soerensen's standard phosphate and Walpole's standard acetate mixtures of known hydrogen ion concentration.

The N/50 acid used in the titrations was prepared by weight from a standard HCl prepared by Hulett and Bonner's (1909) method and checked by gravimetric silver chloride determinations.

Hasselbalch and Gammeltoft (1915) report that freshly platinized electrodes are essential. Our experience entirely corroborates this observation. The electrodes were always carefully cleaned before each determination in accordance with the following routine. The washed electrodes were placed as cathode in the reducing vessel containing 5 per cent H_2SO_4 for 3 minutes where the bubbling hydrogen removed a considerable quantity of precipitated protein that could not be washed off. Then they were rinsed with water, placed as cathode in the platinic chloride solution for 2 minutes, rinsed, returned to the reducing vessel for 3 minutes, rinsed thoroughly with tap water, and finally with distilled water. The electrodes were often checked against the standard solutions.

Calculation of Results.

All H^+ concentrations are expressed by Soerensen's symbol pH, the negative Briggs logarithm of the hydrogen ion concentration. pH values are more easily visualized and plotted than those of C_H, the actual hydrogen ion concentration. The equation for the calculation of results was:

$$\log \frac{1}{C_H} = pH = \frac{E - 0.337}{0.0577\,[1 + (t^\circ - 18^\circ)\ 0.0002]}$$

In our work the variations in potential and temperature were such that it was most convenient to construct tables giving pH directly from the observed readings. We used the rounded value of 0.337 for the N/10 calomel electrode adopted by the Potential Commission (Auerbach).

Preparation of Solution.

All our determinations have been made on plasma rather than whole or defibrinated blood; because removal of the corpuscles eliminates the greater part of the oxygen and consequently allows much quicker reduction of the solution by the hydrogen electrode.

The blood was drawn into a tube containing a small amount of powdered potassium oxalate (always less than 0.5 per cent) to prevent clotting, centrifuged at once, and the plasma pipetted off. Experiments proved that the oxalate had no effect on the determination. If it was impossible to run the determination at once, the plasma was placed in a stoppered paraffined tube in the refrigerator.

In comparing the alkaline reserve of different plasmas by titration with acid, two methods are available. First, the amount of acid required to bring the plasma to a definite H^+ concentration may be determined by a series of determinations and consequent plotting of the titration curve. Or, second, the H^+ concentration resulting from the addition of an arbitrarily fixed amount of acid may be found. This is the most economical, both of time and blood, the deciding considerations when series of observations on a number of patients are desired. We therefore used the former only to select suitable conditions to use in this investigation.

However, since the initial reaction of all plasmas is practically constant and the titration curve is almost a straight line, it is entirely possible to construct the curve from one determination and from it determine the amount of acid required to bring the plasma to any desired reaction.

It is evident from the analysis of the equilibrium $\frac{H_2CO_3}{NaHCO_3}$ that all samples must be brought to a definite CO_2 content. The conditions under which the blood is drawn, the time elapsing before determination, etc., are so variable that the plasma cannot be used as it is. Its CO_2 content must either be fixed under definite conditions, or the CO_2 must be removed.

Two procedures have been followed. The first was worked out for and used throughout the investigation of the first series of experiments on diabetic acidosis; the second was developed on the basis of experience with that series.

Technique of Determination.

Procedure A.—It was found that two successive shakings in an evacuated vessel, as described in the next paragraph, reduced the CO_2 content to a fairly constant level. Experiments with

N/50 HCl showed that more than three volumes of this acid per volume of plasma precipitated some of the proteins. The addition of one volume of N/50 acid to one volume of plasma (Fig. 1) brought normal plasma to about the neutral point. Plasma with less than normal alkaline reserve would, under the same conditions, fall on the acid side of the neutral line.

The oxalated blood was centrifuged and the plasma pipetted off. About 3 cc. of the plasma were placed in a 300 cc. separatory funnel, which was evacuated by means of a water aspirator to a pressure of about 20 mm. The funnel was then rotated for 3 minutes to insure maximum surface and permit the escape of carbon dioxide from the solution. Air was then admitted, the funnel was evacuated, and was again rotated for 3 minutes. Exactly 1 cc. of this plasma was then measured into a 3 cc. dropping funnel, and 1 cc. of N/50 HCl added (calibrated 1 cc. pipettes were used for both acid and plasma); the funnel was closed by means of a small rubber stopper and the solution mixed, without shaking. The mixed solution was then allowed to flow through a small piece of rubber tubing into the Clark electrode, displacing part of the hydrogen with which the vessel had been filled. The care taken in mixing and introducing the acidified solution was to prevent loss of CO_2. The vessel was then shaken for 3 or 4 minutes, the electrode adjusted to minimum contact, and the potential read. The vessel was shaken again 3 or 4 minutes, a fresh contact with the saturated KCl solution was made, another reading taken, and the process was continued until equilibrium was reached, which usually was at the second or third reading.

Procedure B.—The method outlined under A is open to the objection that careful attention is required to bring the carbon dioxide to the arbitrary level. This possibility of error can be eliminated by adding enough acid to complete the reaction $NaHCO_3 + HCl = NaCl + H_2CO_3$, and then removing all the carbon dioxide by evacuation. The carbon dioxide tension of the plasma is thus eliminated as a factor in the results, and the hydrogen ion concentration is dependent on the equilibrium between the plasma buffers, chiefly the proteins, and the excess of acid not neutralized by bicarbonate. It was found that the addition of two volumes of N/50 HCl to one of plasma was sufficient to effect complete decomposition of the bicarbonate, and the re-

sulting solution after removal of the carbon dioxide showed a C_H of about $10^{-5.6}$ (pH = 5.6). At this point the equilibrium in the electrode vessel is obtained much quicker than at the neutral point. This procedure has, over A, the advantages of greater rapidity, the use of smaller amounts of plasma, and the elimination of variations in the carbon dioxide content as a source of error. It has the disadvantage, compared with procedure A, in that the differences in pH corresponding to given differences in "alkaline reserve" are not so great.

In procedure B two volumes of N/50 HCl are added to one of plasma (the amounts being usually 2 and 1 cc. respectively) in a small separatory funnel, 25 to 100 cc., the solution is exhausted with a water aspirator to about 20 mm., and agitated for 1 minute. One exhaustion completely removes the carbon dioxide. The solution is then transferred to the electrode vessel by a pipette, allowed to displace part of the hydrogen, and the reading obtained in the same manner as in A.

EXPERIMENTAL.

The Determination of the Optimum Amounts of N/50 Acid.

Samples of oxalated plasmas were exhausted twice with the water aspirator and the H^+ of the exhausted plasma determined. Then a series of determinations was made varying the ratio of acid and plasma volume as shown in Fig. 1.

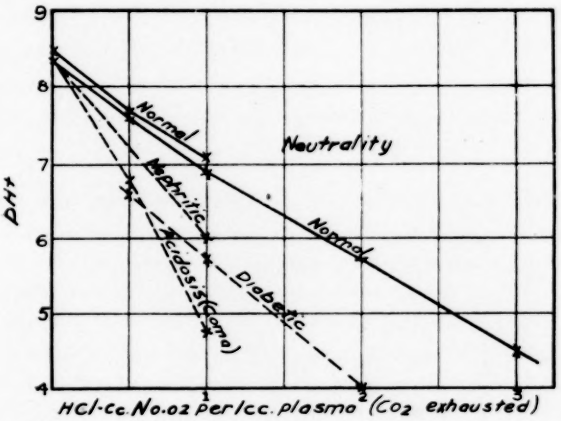

FIG. 1.

It is evident that the addition of one volume of N/50 acid to one volume of plasma brings normal plasma to about the neutral point. The acidosis plasma affords an extreme example of diminished alkaline reserve.

Determination of the Number of Exhaustions Required in Water Aspirator.

Samples of plasma were saturated with alveolar air (5.5 per cent CO_2), portions were placed in separatory funnels, and exhausted on a water aspirator to about 20 mm. for periods of 3 minutes each. During the exhaustion the funnels were rotated constantly to expedite the escape of the CO_2 from the solution. Air was admitted to the funnels between exhaustions. 1 cc. portions were then mixed with 1 cc. portions N/50 HCl in 3 cc. dropping funnels, as described above, and the contents run into the electrode vessels (Table I).

TABLE I.

Number of Exhaustions Needed for Procedure A. pH after Adding One Volume N/50 HCl to Exhausted Plasma.

Human plasma.	Treatment of sample.			
	Saturated with 5.5 per cent CO_2.	Exhausted once.*	Exhausted twice.	Exhausted three times.
	pH	*pH*	*pH*	*pH*
1	6.42	6.67	6.69	6.69
2	6.66	6.76	6.78	6.78
3	6.47		6.63	6.63
	6.45		6.62	6.62
4		7.03	7.03	7.03

* Each exhaustion lasted 3 minutes.

It appeared from the above that two exhaustions were sufficient for our purpose, and it was desirable to avoid as far as possible concentrating the plasma by evaporation. Later, however, the perfection of Van Slyke's CO_2 apparatus furnished an accurate and convenient means of checking the above experiments. The amounts of carbon dioxide remaining after successive exhaustions were determined. The plasma was placed in large separatory funnels, the exhaustions were carried out as described under pro-

cedure A, and a 1 cc. sample was removed for analysis after each exhaustion (Table II).

TABLE II.

Number of Exhaustions Needed for Procedure A. Total Residual CO_2 Determined by Van Slyke's Method. Corrected to 0°, 760 Mm.

Human plasma.	Saturated with 6.3 per cent CO_2.	CO_2 remaining after									
		1st 3′ exhaustion.	Per cent of CO_2 at saturation.	2nd 3′ exhaustion.	Per cent of CO_2 at saturation.	3rd 3′ exhaustion.	Per cent of CO_2 at saturation.	4th 3′ exhaustion.	Per cent of CO_2 at saturation.	6th 3′ exhaustion.	Per cent of CO_2 at saturation.
	cc.	cc.		cc.		cc.		cc.		cc.	
G.....	0.79	0.60	76.2	0.54	68	0.49	62	0.48	60	0.45	57
A.....	0.72	0.53	73	0.43	60	0.40	56	0.38	53	0.38	51
An.....	0.75	0.55	73	0.45	60	0.41	54	0.38	51		
O.....	0.775	0.57	74	0.52	67	0.46	59	0.43	55	0.40	51

It is evident from Table II that exhaustion was not complete. This was expected. However, the variations after two exhaustions were so small in their effect on pH, as demonstrated in Table I, that accurately reproducible results are obtainable when two exhaustions, carried out as described, are used as the arbitrary standard.

That the arbitrarily selected double exhaustion approximates to a constant carbon dioxide content is also evident from the curves constructed from these values in Fig. 2.

The carbon dioxide as a source of error has been entirely eliminated in procedure B by the use of larger amounts of acid and exhaustion *after* its addition. The following experiment shows this (Table III).

Two volumes of N/50 acid were added to one volume of plasma in a separatory funnel. The funnel was exhausted to about 20 mm. for 1 minute, with constant rotation. 1 cc. was taken for analysis and the exhaustion repeated.

The amounts of residual gas in column 3 are entirely comparable with the "dissolved air" correction in this method; therefore no measurable amounts of carbon dioxide were present.

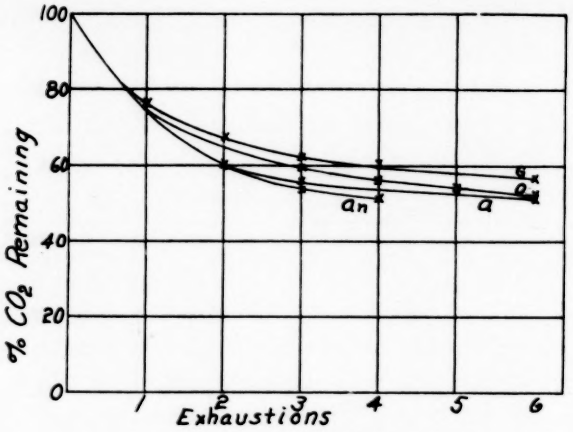

FIG. 2. From Table II.

TABLE III.

Number of Exhaustions in Water Aspirator Needed after Adding Two Volumes of N/50 HCl. Dissolved Gases Determined by Van Slyke's Method. 30° and 762 Mm.

Sample plasma.	Exhausted 3′ with shaking. Gas observed.	Dissolved air calculated as present.	CO_2 present.
	cc.	*cc.*	*cc.*
Mixed normal	0.04	0.044	0.000
Same saturated with 6.3 per cent CO_2	0.04	0.044	0.000
A	0.045	0.044	0.000
Dog	0.04	0.044	0.000
H	0.05	0.044	0.000

Comparison of Two Procedures for Removing Carbon Dioxide.

Hydrogen ion concentrations were determined on two plasmas. Each was then divided in two portions, one of which was placed in a separatory funnel and was exhausted 3 minutes as in procedure A. 1 cc. samples were then mixed with various volumes of N/50 acid and the resulting reaction was determined.

To samples of the other portions in separatory funnels varying volumes of N/50 acid were added and the mixtures exhausted

for 1 minute. The results are given in Table IV and plotted in Figs. 3 and 4; it is evident that the two series differ but little.

TABLE IV.

Comparison of Procedures A and B.

Plasma.	N/50 acid per cc. plasma.	A (exhausted twice before adding acid).	B (exhausted once after adding acid).
	cc.		
P.			
pH = 8.63.	0.5	7.73	7.56
		7.74	7.58
	1.0	6.74	6.68
		6.74	
	2.0	5.43	5.34
		5.42	5.33
	3.0	4.54	4.40
		4.53	4.40
M.			
pH = 8.61.	0.5	7.76	7.83
	1.0	6.88	6.87
	2.0	5.47	5.48

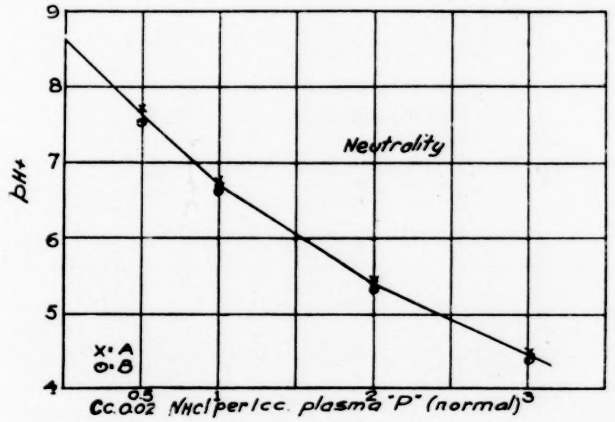

FIG. 3.

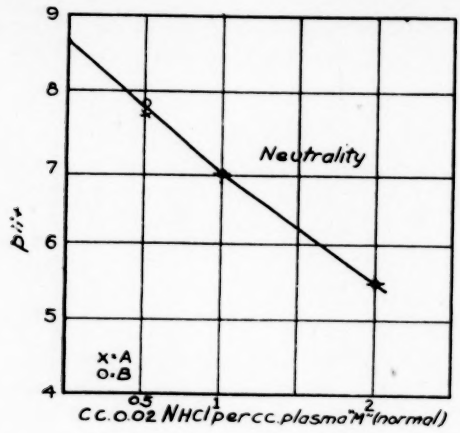

FIG. 4.

Experiment to Determine the Effect of Oxalate on the Titration of Plasma.

Solutions of M/10 phosphates and of normal plasma were made up with varying concentrations of potassium oxalate. H^+ concentration determinations were made as indicated in Table V. It is evident that in plasma oxalate up to 0.5 per cent does not produce an appreciable change and that the change produced by 1 per cent is within the allowable limits of experimental error. About 0.2 to 0.3 per cent is ordinarily sufficient to prevent clotting.

TABLE V.

Effect of Oxalate.

On pH of M/10 phosphate solution concentration.		On pH of M/10 phosphate solution + 1 vol. N/50 HCl concentration.		On pH of normal plasma treated by procedure B (2 vol. N/50 acid added) concentration.	
K oxalate.	pH.	K oxalate.	pH.	K oxalate.	pH.
per cent		*per cent*		*per cent*	
0	7.50	0	6.74	0	5.38
0.2	7.47	0.25	6.74	0.5	5.38
0.6	7.47	0.5	6.74	1.0	5.39
1.0	7.44	1.0	6.72	2.0	5.45
1.6	7.45			4.0	5.53
2.0	7.94				

Effect of Renewing Solutions in Electrodes.

Hasselbalch pointed out the possibility of renewing successively the solutions in the electrode without renewing the H_2 as a means of bringing the CO_2 content of the electrode gas to equilibrium with that of the solution. The following experiment (Table VI) shows that this precaution is unnecessary with the small amount of residual CO_2 present under procedure A.

TABLE VI.

Effect of Renewing Solution without Change of H_2 Atmosphere. New Solutions Were Run into the Electrode Vessel Displacing the Used Solution without Changing the H_2 Bubble.

Plasma.	E.	S.	U.	H.	M.	M. 2.	V.
pH of 1st determination...	8.13	7.11	6.48	7.03	6.55	6.89	6.99
pH of renewed solution....	8.14	7.11	6.48	7.03	6.58	6.91	6.99

Accuracy of the Method.

Clark has pointed out the possibility of accuracy attainable with his electrode. With plasma we have considered 1 millivolt = 0.02 pH our permissible error. This amount is very small in comparison with the gross differences we have obtained in the clinical studies.

Table VII gives samples of the results obtained in duplicate determinations.

Titration of Plasma Containing Lactic and β-Oxybutyric Acids.

Since a diminished alkaline reserve is the result of the introduction of abnormal acid products of metabolism, it seemed desirable to investigate the effects of adding such acids to a normal plasma *in vitro*. The results of such experiments are shown in Tables VIII and IX, and Figs. 5 and 6. These experiments were performed as follows:

Experiment I. Curves A and B.—A weighed amount of acid was added to a portion of plasma to make the plasma 0.2 per cent acid solution. This solution was then mixed with untreated plasma in varying proportions and the resulting mixtures were

TABLE VII.

Duplicate Determinations Made with Different Electrodes in Different Vessels.

Procedure A.					Procedure B.				
Plasma (human).	Date.	Temperature.	Reading	pH.	Plasma.	Date.	Temperature.	Reading	pH.
		°C.	millivolts				°C.	millivolts	
A.	May 15	23	723	6.57	Dog VIII.		25	630	4.96
		23	722	6.55			23	627.7	4.96
W.	" 25	24	742	6.87	"	June 14	25	627	4.91
Normal.		23.5	740	6.85			23	626.6	4.93
V.	" 26	23	744	6.93	V.* (human).	" 13	25	650.4	5.30
Normal.		23	744	6.93			26	652.5	5.33
							26	651.6	5.31
M.	" 23	24	749	6.99			26	651.6	5.31
Normal.			749.5	7.00			26.5	652.3	5.32
								652.2	5.32
H.	March 21	20	723	6.64					
		21	723	6.62	Dog 2 a.	" 10	25	620	4.98
							26	622.6	4.91
					" 2 b.	" 10	25	631.5	4.98
							26	632	4.97
					" 3.	" 11	22	630	5.00
								630.1	5.00

* These six determinations were run with six distinct pipettes, funnels, and electrodes, and in two electrode vessels.

titrated by both A and B methods. The acids were analyzed by adding excess N/7 NaOH, warming on a steam bath for a couple of hours, and titrating back with N/7 HCl using phenolphthalein.

	per cent
β-Hydroxybutyric acid analyzed	100
Lactic acid analyzed	95.2

Experiment II. Curve C.—The acids used in the preceding experiment had not been treated to remove any anhydride that might have been present; the experiment was therefore repeated.

TABLE VIII.

Effect of Organic Acids on the Titration Curve.

Normality of organic acid on basis of plasma volume only.	A 1 vol. plasma + 1 vol. N/50 acid. Curve A.	B 1 vol. plasma + 2 vol. N/50 acid. Curve B.
	β-Hydroxybutyric acid (Fig. 5).	
	pH	*pH*
0	6.73	5.34
	6.72	5.35
0.0094	6.55	5.16
	6.55	5.16
0.024	5.84	4.85
	5.84	4.85
0.047	5.08	4.58
	5.07	4.57
0.094	4.59	4.29
	4.58	4.28
0.189	4.15	4.00
		4.00
	Lactic acid (Fig. 6).	
0.011	6.26	4.94
	6.24	4.94
0.028	5.355	4.565
	5.345	4.575
0.056	4.58	4.18
		4.18
0.11	4.03	3.83
	4.02	3.83
0.22	3.64	3.48
	3.65	3.48

0.225 gm. of lactic acid was placed in a flask with 30 cc. of N/10 sodium hydroxide, placed on the steam bath for 2 hours, 50 cc. of N/10 HCl were added, and the solution was diluted to 100 cc. The solution was then N 0.025 to lactic acid and N 0.02 to hydrochloric acid.

2 gm. of CaZn β-oxybutyric acid salt were treated with three-fourths of its equivalent sulfuric acid and the liberated acid was extracted with ether in a wet extractor. The ether was evaporated off over water and the solution diluted to 100 cc. The amount of acid was titrated with sodium hydroxide as in lactic

acid. 0.130 gm. of this acid was then treated with 15 cc. of N/10 sodium hydroxide heated on the steam bath for 2 hours, 25 cc. of N/10 HCl were added, and the solution was diluted to 50 cc. This solution was then N 0.025 to β-oxybutyric acid and N 0.02 to hydrochloric acid.

TABLE IX.

Effect of Organic Acid on the Titration Curve.

Solution A. 1 vol. plasma + 2 vol. N/50 HCl.	Solution B.*	Normality of organic acid on basis of plasma volume only.	Lactic acid. Curve C, Fig. 6.	β-Hydroxybutyric acid. Curve C, Fig. 5.
			pH	*pH*
—	0	0	4.55	4.55
4.0	0.5	0.0055	4.41	4.45
4.0	1.0	0.01	4.32	4.39
4.0	2.0	0.0166	4.20	4.31
3.0	3.0	0.025	4.09	4.24
1.0	3.0	0.037	3.96	4.15
0	—	0.05	3.85	4.07

* Solution: β-oxybutyric acid or lactic acid, N 0.025; hydrochloric acid, N 0.02.

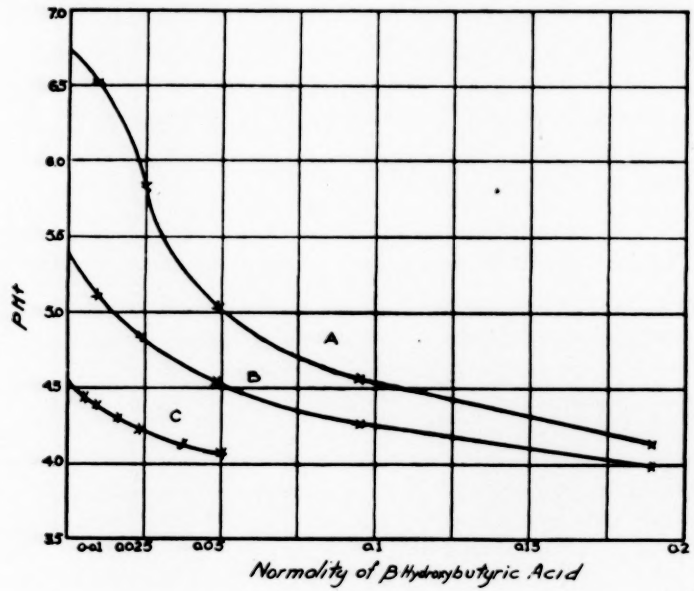

FIG. 5.

The normality figures in A and B do not represent actual amounts of free acid due to the presence of undissociated anhydride, but they do indicate the change in the titration curve caused by the accumulation of such acid. Curve C indicates the nature of the decreased reserve in a dog plasma with very low reserve. The normality figures here represent actual values.

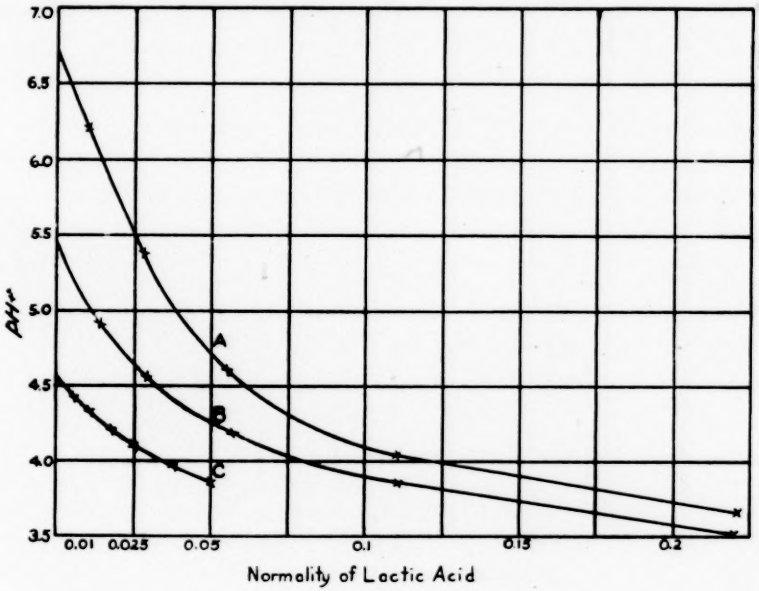

FIG. 6.

Range of Normal Values.

Table X shows the variations in normal plasma in different individuals at different times. The values in a healthy man may change on titration with one volume of N/50 acid from a pH of 6.75 to 7.00 during the course of the day. The corresponding figures for titration with two volumes of N/50 acid are from a pH of 5.60 to 5.90. Dogs have a lower reserve averaging around a pH of 5.00.

The normal base line for titration with one volume of N/50 acid in the accompanying charts is taken at 7.00; with two volumes at 5.6. The normal limits for the "carbon dioxide combin-

ing capacity" is from 55 to 70. The normal alveolar carbon dioxide tension in mm. is about 38. From a number of comparisons the ratio between plasma carbon dioxide volume per cent and alveolar carbon dioxide tension in mm. has been established as 1.45 (see Paper VI).

TABLE X.

Table of Normal Variations.

Procedure A. 1 vol. plasma + 1 vol. N/50 acid.			Procedure B. 1 vol. plasma + 2 vol. N/50 acid.		
Plasma.	Time.*	pH.	Plasma.	Time.	pH.
M.	a. m.	6.86	Dog 1.	a. m.	4.81
	p. m.	6.80		p. m.	5.02
V.	May 15, a. m.	7.04	" 2.	a. m.	5.18
	p. m.	6.89		p. m.	5.24
	" 17, a. m.	6.96			
	p. m.	6.94	" 3.	a. m.	4.91
				p. m.	5.23
D.	a. m.	7.10			
	p. m.	7.60	M.	May 18	5.56
				" 22	5.63
P.	a. m.	6.73			
	p. m.	6.84	F.		5.69
S.	a. m.	6.65	P.		5.50
	2 p. m.	6.74			
	4 "	6.71			
	6 "	6.82			
C.	May 19, a. m.	6.58			
	p. m.	6.83			
	"	6.83			
	" 22, p. m.	6.85			

* Indicates before and after meals.

Application of the Method.

The charts of diabetic patients followed for a long period by the electrometric titration of plasma, simultaneously with the determination of CO_2 combining capacity of blood and plasma, alveolar air, and acid excretion, are given in Paper VI of this series.

The values of simultaneous determination of the "carbon dioxide combining capacity" and alveolar carbon dioxide are also shown, since it was one purpose of this series of observations to select the most suitable method for determining the actual amount of "alkaline reserve" in the body.

The samples of blood were obtained from the arm, were drawn through a McRae needle into tubes containing a small amount of potassium oxalate, and centrifuged at once. The determinations were, with a few exceptions, done at once, but samples may be kept for a considerable time in paraffined tubes.

The observations were divided into two periods; the first covering the spring of 1915, the second that of 1916. The electrometric titrations on the first series were done by procedure A — 1 volume of plasma + 1 volume of N/50 HCl; on the second series by procedure B — 1 volume of plasma + 2 volumes of N/50 HCl. This eliminated the theoretical objection of possible error due to the presence of carbon dioxide, but proved to have the disadvantage of lessening the sensitivity of the titration, in that for given decrease in carbon dioxide combining power the corresponding change in pH was less than with one volume of acid.

CONCLUSIONS.

The conclusions are evident from the charts. The two methods of determining the "alkaline reserve" are entirely comparable and furnish a reliable index of the reserve actually existing in the body. The titration of the plasma includes the influence of all the "buffers," not only the sodium bicarbonate, but also the proteins and the minute amounts of phosphates. The fact that the electrometric titration of the plasma gives results parallel with the carbon dioxide combining capacity indicates that the latter is proportional to the total "buffer" content of the plasma.

SUMMARY.

1. The gas chain method of determining hydrogen ion concentration has been utilized in the titration of plasma and the conditions for successful operation have been determined.

2. Values for normal and pathological plasmas have been determined.

3. The method has been compared with that of the carbon dioxide combining capacity in a long series of diabetic patients, and the close agreement of the two methods has been established.

BIBLIOGRAPHY.

Auerbach, F., *Z. Electrochem.*, 1912, xviii, 13.
Bjerrum, N., *Samml. Chem. u. Chem.-Tech. Vortr.*, 1915, xxi, 1.
Benedict, H., *Arch. ges Physiol.*, 1906, cxv, 106.
Clark, W. M., *J. Biol. Chem.*, 1915, xxiii, 475.
Clark, W. M., and Lubs, H. A., *J. Biol. Chem.*, 1916, xxv, 479.
Ellis, J. H., *J. Am. Chem. Soc.*, 1916, xxxviii, 737.
Fridericia, L. S., *Berl. klin. Woch.*, 1914, li, 1268.
Hasselbalch, K. A., *Biochem. Z.*, 1911, xxx, 317; 1913, xlix, 415; *Compt. rend. Lab. Carlsberg*, 1911, x, 69.
Hasselbalch, K. A., and Gammeltoft, S. A., *Biochem. Z.*, 1915, lxviii, 205.
Hildebrand, J. H., *J. Am. Chem. Soc.*, 1913, xxxv, 847.
Hoppe-Seyler, F., and Thierfelder, H., Handb. physiol. u. path. Chem. Analyse, Berlin, 7th edition, 1903.
Hulett, G. A., and Bonner, W. D., *J. Am. Chem. Soc.*, 1909, xxxi, 390.
Hulett, *Physical Rev.*, 1911, xxxiii, 307.
von Jaksch, R., *Z. klin. Med.*, 1888, xiii, 350.
Lipscomb, G. F., and Hulett, G. A., *J. Am. Chem. Soc.*, 1916, xxxviii, 20.
Loomis, N. E., and Acree, S. F., *Am. Chem. J.*, 1911, xlvi, 585.
Lundsgaard, C., *Biochem. Z.*, 1912, xli, 247.
Magnus-Levy, A., *Arch. exp. Path. u. Pharm.*, 1899, xlii, 149.
McLean, F. C., and Van Slyke, D. D., *J. Am. Chem. Soc.*, 1915, xxxvii, 1128.
Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.
Peabody, F. W., *Arch. Int. Med.*, 1914, xiv, 236.
Soerensen, S. P. L., *Ergebn. Physiol.*, 1912, xii, 293.
Stillman, E., Van Slyke, D. D., Cullen, G. E., and Fitz, R., *J. Biol. Chem.*, 1917, xxx, 405.
Walpole, G. S., *J. Chem. Soc.*, 1914, cvi, 2501.
Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.
Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.

STUDIES OF ACIDOSIS.

IV. THE RELATIONSHIP BETWEEN ALKALINE RESERVE AND ACID EXCRETION.

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The object of the work presented in this paper was to ascertain whether in diabetes a quantitative relationship could be discovered between the alkaline reserve of the blood plasma, as measured by its combining power for CO_2, and the rate of acid excretion by the kidneys. It has been known since the time of Walter[1] that entrance of acid into the circulation immediately reduces the blood bicarbonate, and is accompanied by an increased rate of acid and ammonia excretion in the urine. A quantitative relationship between decreased blood bicarbonate and acid excretion has not been demonstrated.

To undertake this problem the plasma bicarbonate was estimated in volumes per cent by the method described in Paper I of this series and compared with the excretion of acid in the urine in the following way. The ammonia of the urine was added to the acid titratable with phenolphthalein by Folin's method[2] as a measure of the excretion of acids in excess of mineral bases, the whole being expressed as 0.1 N acid. The greater part of such excess acid is usually neutralized by ammonia in man, but as shown by Henderson and Palmer[3] an acid as weak as β-hydroxybutyric can be excreted 45 per cent free, while more than 80 per cent of the phosphate can be excreted as the acid NaH_2PO_4. Consequently in order to determine the total excretion of acid in excess of mineral bases the free titratable acid must be added to that neutralized by ammonia.

[1] Walter, F., *Arch. exp. Path. u. Pharm.*, 1877, vii, 148.

[2] Folin, O., *Am. J. Physiol.*, 1903, ix, 265.

[3] Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1913, xiv, 81.

The Folin method for titration of free acid was chosen because the acid titratable with phenolphthalein approaches zero in human urine when the height of the plasma bicarbonate is at its maximum normal of about 80 volume per cent, under which conditions ammonia excretion also approaches zero.

Comparison of the amounts of ammonia plus acid excreted per 24 hours in a number of diabetics followed over considerable periods in the Rockefeller Hospital indicated a general relationship between daily excretion and the plasma bicarbonate content. It was impossible, however, to form any estimate of the plasma bicarbonate from the rate of excretion alone, and the ordinary $\frac{NH_3}{\text{total N}}$ ratio was even less satisfactory. As Folin showed, this ratio may be greatly increased beyond the usual value by merely reducing the protein intake and consequently the denominator of the ratio; and quite aside from variations that could be explained by this cause, the ammonia ratio bore only the most casual quantitative relationship to the plasma bicarbonate.

In the meantime, however, one of us (F.) working on diabetic metabolism at the Peter Bent Brigham Hospital had used the Ambard formula to compare the acid excretion with the blood alkali as indicated by the alveolar CO_2 tension. Ambard and Weill[4] found that in the cases of urea and chloride, excretion rate is proportional to the square of the concentration of the excretory substances in the blood above the excretion threshold, to the square root of the volume of water eliminated, and to the first power of the weight of the subject. (For a complete discussion see McLean.[5]) The above facts are reduced to algebraic form by the equation:

$$(1)\quad \text{Blood concentration} = \text{constant} \times \sqrt{\frac{D}{W}} \sqrt{C}$$

D is the excretion rate, W the body weight, and C the concentration of excretory product in the urine. The expression $\sqrt{\frac{D}{W}} \sqrt{C}$ is proportional to the blood concentration of the excretory product above the excretion threshold.

[4] Ambard, L., Physiologie normale et pathologique des reins, Paris, 1914.

[5] McLean, F. C., *J. Exp. Med.*, 1915, xxii, 212.

If acid excretion should follow the same law, a similar expression might be expected to indicate the degree of acid retention in the blood. In order to test this possibility, the rate of excretion of 0.1 N acid + NH_3 (24 hour time unit) was taken as D and the cc. of 0.1 N acid + NH_3 per liter of urine as C. Assuming that acid accumulation in the plasma is proportional to the fall of the plasma CO_2 figure below the maximum figure of 80, we may express the relation between the blood accumulation and acid excretion as follows:

$$(2)\quad \text{Retained acid} = 80 - \text{plasma } CO_2 = \text{constant} \times \sqrt{\frac{D}{W}\sqrt{C}}$$

The constant of the equation in repeated observations proved to be 1, so that the relationship between plasma CO_2 capacity and acid excretion may be expressed more simply as:

$$(3)\quad \text{Plasma } CO_2 \text{ capacity} = 80 - \sqrt{\frac{D}{W}\sqrt{C}}$$

This formula is purely empirical, and it was not adopted until tests upon some scores of urines had shown that it held more consistently than any other expression which could be found in the literature or devised. As stated before, the rate of excretion alone, as well as the ammonia : total nitrogen ratio failed to show consistent relationship to the blood bicarbonate. No other equation including excretion rate and concentration was so satisfactory as Ambard's. If the square root of the whole were not taken the urine index varied much more than the blood. If the expression $\sqrt{\frac{DC}{W}}$ were used, the influence of concentration (C) was given too much weight, while $\sqrt{\frac{D}{W}\sqrt[3]{C}}$ gave too little influence to C. In short, although reasoning by analogy led to the trial of the Ambard equation, it has been retained solely because it expresses the relationship between blood alkali and acid excretion more accurately than any other expression at present available. The fact that with a given blood bicarbonate concentration $\sqrt{\frac{D}{W}\sqrt{C}}$ is constant shows that, other factors being the same, the amount of acid excreted in excess of mineral

bases is increased, on the average, as the square root of the volume of urine; *i.e.*, increasing the water output four times doubles the rate of acid elimination.

As in Ambard's tests, the value of D and C may be determined upon a 24 hour specimen of urine or upon a shorter period from which the 24 hour rate of excretion can be computed. In the cases reported in this paper short periods were chosen, varying in length from 1 to 4 hours. The advantage of short periods in tests of this description has been pointed out by McLean. By collecting the urine over a given period and withdrawing blood at the middle of it, the blood sample may be assumed to represent the average for the period. If no food or water is taken during the period, and the latter is not too soon after a heavy meal, the rate of excretion during the period will remain practically constant.

Experiments were conducted upon normal individuals to determine the degree of constancy of the relationship expressed in Equation 3 above. Different normal individuals were tested at different times, and a few were given sodium bicarbonate to discover at what level of blood bicarbonate content, if any, the excretion of acid in the urine ceased. The results of these observations are tabulated in Table I.

A certain margin of error must be accepted in calculating the internal concentration of any substance from the excretion, and the margin in this instance appears to be about 10 volumes per cent of plasma CO_2. In the normal cases the maximum variations encountered were 8.7 volumes per cent too high, and 6.7 volumes per cent too low.

Thirty-six cases of diabetes were tested in the manner described, selected as they reported to the hospital. Nothing was known about their previous treatment or condition except that a few stated that they had been taking sodium bicarbonate before the test was made. The results are tabulated in Table II.

Of the 36 cases, 26 showed values of the index from which the plasma CO_2 could be estimated with an error not greater than 10 volumes per cent. Of the remaining 10, 4 showed normal plasma CO_2, the CO_2 calculated from the urine being merely a higher normal. Six cases of acidosis remain in which an error greater than 10 volumes per cent was made in estimating the

TABLE I.

Excretion of Acid Compared with the Plasma Bicarbonate in Normal Individuals.

Subject.	Date.	Time.	Weight	Urine measured.	Volume calculated to 24 hrs.	0.1 N NH_3 per liter.	0.1 N acid per liter.	C	D	$\sqrt{\frac{D}{W}\sqrt{C}}$	CO_2 bound by 100 cc. of plasma.		Difference.	$NaHCO_3$.
											Found.	Calculated by $80-\sqrt{\frac{D}{W}\sqrt{C}}$		
		min.	*kg.*	*cc.*	*cc.*	*cc.*	*cc.*				*cc.*	*cc.*		*gm.*
McL.	Feb. 19, 1916	60	81	285	6,850	70	90	160	1,096	13.1	62.7	66.9	+4.2	
F.	Oct. 28, 1915	120	70	130	1,560	100	266	366	571	12.6	65.0	67.4	+2.4	
P.	Dec. 1, 1915	72	89	34	680	592	415	1,007	684	15.6	65.8	64.4	−1.4	
S.	Nov. 16, 1915	66	86	49.5	1,080	387	416	803	867	16.9	68.3	63.1	−5.2	
F.	Dec. 1, 1915	72	70	84	1,680	252	110	362	652	13.3	69.5	66.7	−2.8	
McL.	Jan. 27, 1916	60	81	375	9,000	45	24	69	621	2.5	69.8	77.5	+7.7	
G.	Oct. 28, 1915	72	82.8	65	1,300	110	100	210	273	6.9	71.6	73.1	+1.5	
V. S.	Nov. 13, 1915	30	70	27	1,297	76	40	116	150	4.8	72.5	75.2	+2.7	3
McL.	Jan. 27, 1916	60	81	167	4,000	19	64	83	332	1.9	73.9	78.1	+4.2	17
V. S.	Nov. 16, 1915	60	70	70	1,440	135	28	163	235	6.5	75.2	73.5	−1.7	
V. S.	Feb. 21, 1916	60	70	—	—	—	—	—	—	11.7	75.3	68.6	−6.7	
V. S.	" 21, 1916	60	70	—	—	—	—	—	—	3.0	78.0	77.0	−1.0	5
McL.	" 19, 1916	60	81	68.5	1,645	6	0	6	10	0.6	79.3	79.4	+0.1	20

TABLE II.

Excretion of Acid Compared with the Plasma Bicarbonate in 36 Cases of Diabetes.

No.	Subject.	Date.	Time.	Weight.	Urine measured.	Volume calculated to 24 hrs.	0.1 N NH_3 per liter.	0.1 N acid per liter.	C	D	$\sqrt{\frac{D}{W}\sqrt{C}}$	CO_2 bound by 100 cc. of plasma. Found.	CO_2 bound by 100 cc. of plasma. Calculated by $80-\sqrt{\frac{D}{W}\sqrt{C}}$	Difference.
				kg.	*cc.*	*cc.*	*cc.*	*cc.*				*cc.*	*cc.*	
1	Z.	Mar. 14, 1917	4 hrs.	23.0	590	3,540	400	244	644	2,280	50.2	14.0	29.8	+15.4
2	C.	June 23, 1916	72 min.	37.3	173	3,460	825	328	1,153	4,000	60.3	22.5	19.7	− 2.8
3	H.	" 10, 1916	72 "	42.0	140	2,800	1,160	280	1,440	4,040	60.4	29.2	19.6	− 9.6
4†	S.	Feb. 15, 1917	72 "	43.0	175	3,500	1,070	430	1,500	5,250	68.8	31.0	11.2	−19.8
5	E.	Nov. 18, 1915	72 "	52.2	175	3,500	900	320	1,220	4,270	53.4	33.6	26.6	− 7.0
6	F.	Dec. 21, 1915	2 hrs.	45.0	500	6,000	252	335	587	3,522	43.5	33.6	36.5	+ 2.9
7	H.	Nov. 22, 1916	72 min.	50.0	306	6,120	612	247	859	5,260	55.5	34.0	24.5	− 9.5
8†	W.	" 21, 1916	72 "	50.0	175	3,500	1,056	431	1,487	5,200	63.3	35.0	16.7	−18.3
9	L.	Feb. 1, 1917	72 "	43.8	535	10,700	160	122	282	3,018	34.0	36.5	46.0	+ 9.5
10†	O'R.	" 14, 1917	72 "	32.8	145	2,900	1,116	438	1,554	4,500	73.5	41.0	6.5	−34.5
11	A.	Sept. 6, 1916	72 "	42.2	285	5,700	374	266	640	3,642	46.7	42.1	33.3	− 8.8
12	L.	June 8, 1916	72 "	50.0	127	2,540	671	352	1,023	2,600	40.8	42.8	39.2	− 3.6
13	O'B.	Feb. 23, 1917	72 "	37.2	56	1,120	1,088	496	1,584	1,775	43.6	47.0	36.4	−10.6
14	M.	Aug. 24, 1916	72 "	37.0	104	2,080	450	402	852	1,770	37.4	47.2	42.6	− 4.6
15	C.	Nov. 11, 1915	2 hrs.	47.0	140	1,680	506	152	658	1,080	24.3	47.8	55.7	+ 7.9
16†	D.	Jan. 2, 1916	72 min.	28.8	118	2,360	1,100	425	1,525	3,600	69.9	47.8	10.1	−37.7
17	C.	Oct. 30, 1915	72 "	60.0	145	2,900	268	260	528	1,531	24.2	50.6	55.8	+ 5.2

18	C.	Aug. 29, 1916	72 min.	52.0	229.5	4,590	—	—	335	1,538	23.3	50.7	56.7	+ 6.0
19	H.	Oct. 11, 1916	72 "	56.1	375	7,500	182	164	346	2,590	29.3	50.8	50.7	− 0.1
20	U.	" 19, 1915	72 "	46.5	87	1,740	668	296	964	1,677	33.5	51.1	46.5	− 4.6
21	P.	Nov. 11, 1915	72 "	46.0	305	6,100	168	64	232	1,415	23.2	52.5	56.8	+ 4.3
22	D.	" 4, 1915	72 "	48.7	110	2,200	172	575	747	1,644	20.4	53.3	59.6	+ 6.3
23	K.	" 11, 1915	72 "	28.5	40	800	468	130	598	478	20.2	53.5	59.8	+ 6.3
24	R.	June 16, 1916	72 "	49.2	270	5,400	88	94	182	985	16.4	54.7	63.6	+ 8.9
25	S.	Dec. 8, 1915	72 "	42.6	83	1,660	144	50	194	320	10.2	55.1	69.8	+14.7
26	D.	Oct. 21, 1915	72 "	50.0	100	2,000	264	316	580	1,160	23.6	55.4	56.4	+ 1.0
27	R.	Nov. 18, 1915	3 hrs.	87.2	360	2,880	328	305	633	1,822	23.0	56.2	57.0	+ 0.8
28	S.	Dec. 1, 1915	2½ "	50.4	520	5,000	172	105	277	1,390	21.4	56.4	58.6	+ 2.2
29	C.	Sept. 28, 1916	72 min.	40.0	600	12,000	171	28	199	2,400	29.1	57.9	50.9	− 7.0
30	S.	" 28, 1916	72 "	51.0	175	3,500	222	146	368	1,290	22.0	57.9	58.0	+ 0.1
31	H.	Nov. 11, 1915	2 hrs.	47.8	340	4,080	44	24	68	278	6.9	58.2	73.1	+14.9
32	P.	" 15, 1915	1¼ "	54.0	100	1,694	120	140	260	441	11.5	59.5	68.5	+ 9.0
33	S.	June 8, 1916	72 min.	25.8	135	2,700	313	50	363	980	26.9	59.8	53.1	− 6.7
34	W.	Aug. 24, 1916	72 "	67.8	42	840	150	129	279	234	6.8	61.0	73.2	+12.2
35	M.	Nov. 11, 1915	2 hrs.	50.2	150	1,800	108	28	136	245	7.5	61.4	72.5	+11.1
36	C.	June 13, 1916	72 min.	39.8	64	1,280	418	64	482	616	18.4	69.2	61.6	− 7.6

*Coma.

†$NaHCO_3$.

TABLE III.

Excretion of Acid Compared with the Plasma Bicarbonate in 29 Observations on the Same Case.

No.	Date.	Time.	Weight.	Urine measured.	Volume calculated to 24 hrs.	0.1 N NH_3 per liter.	0.1 N acid per liter.	C	D	$\sqrt{\frac{D}{W}}\sqrt{C}$	CO_2 bound by 100 cc. of plasma.		Difference.	$NaHCO_3$
											Found.	Calculated by $80-\sqrt{\frac{D}{W}}\sqrt{C}$		
	1915	*min.*	*kg.*	*cc.*	*cc.*	*cc.*	*cc.*							*gm.*
1	Oct. 16	72	48.0	100	2,000	1,242	540	1,782	3,564	56.0	29.0	24.0	− 5.0	
2	" 17	72	48.4	100	2,000	876	332	1,208	2,416	41.6	41.6	39.4	− 2.2	
3	" 19	72	48.7	110	2,200	828	216	1,044	2,297	39.0	43.6	41.0	− 2.6	
4	Nov. 8	72	46.8	200	4,000	204	85	289	1,156	20.5	45.9	59.5	+13.6	
5	" 19	2⅛ hrs.	48.0	59	531	1,352	335	1,687	896	27.7	47.9	52.3	+ 4.4	
6	" 9	72	46.8	130	2,600	448	190	638	1,659	29.9	48.4	50.1	+ 1.7	
7	Oct. 16	1 hr.	48.0	178	4,272	154	312	466	1,991	29.9	48.6	50.1	+ 1.5	50
8	Nov. 22	2 hrs.	48.2	70	774	780	205	985	685	23.7	48.6	56.3	+ 7.7	
9	" 5	72	47.8	55	1,100	912	595	1,507	1,658	36.7	48.9	43.3	− 5.6*	
10	" 12	72	47.0	35	700	1,168	450	1,618	1,133	31.1	49.3	48.9	− 0.4	
11	" 4	72	47.8	40	800	896	480	1,376	1,101	29.2	51.1	50.8	− 0.3	
12	" 13	72	46.7	105	2,100	568	220	788	1,655	31.5	51.5	48.5	− 3.0	
13	" 10	72	47.5	175	3,500	316	130	446	1,561	26.3	52.5	53.7	+ 1.2	
14	Oct. 23	72	49.5	55	1,100	1,057	372	1,429	1,572	34.2	52.7	46.0	− 6.7	
15	Nov. 24	2¼ hrs.	48.6	125	1,333	360	75	435	578	15.7	54.5	64.3	+ 9.8	
16	Oct. 27	72	50.1	85	1,700	400	215	615	1,045	22.7	54.9	57.3	+ 2.4	
17	Nov. 29	72	49.4	18	360	880	300	1,180	425	17.2	55.5	62.8	+ 7.3	
18	" 15	72	46.8	20	400	1,069	240	1,309	428	18.2	55.6	61.8	+ 6.2	
19	" 3	72	48.0	75	1,500	536	280	816	1,224	27.0	55.7	53.0	− 2.7	

20	Oct. 29	72	49.3	470	9,400	72	40	112	1,052	8.4	56.9	71.6	+14.7	
21	" 20	72	49.4	134	2,680	596	200	796	2,134	34.9	57.2	45.1	−12.1	30
22	" 22	72	49.6	291	5,820	230	92	332	1,932	26.6	57.8	53.4	− 4.4	
23	" 25	72	50.5	245	4,900	240	88	328	1,607	24.0	57.9	56.0	− 1.9	
24	" 28	72	50.0	360	7,200	124	80	204	1,469	20.5	58.4	57.5	− 0.9	
25	" 26	72	50.3	220	4,400	164	52	216	948	16.6	59.0	63.4	+ 4.4	
26	" 18	72	49.5	340	6,800	332	168	500	3,400	39.2	60.0	40.8	−19.2	30
27	Nov. 2	72	48.7	30	600	784	380	1,164	698	22.1	60.6	57.9	− 2.7	
28	" 1	72	48.8	130	2,600	148	44	192	499	11.9	60.8	68.1	+ 7.3	
29	Oct. 21	72	50.0	250	5,000	288	128	416	2,080	29.1	64.0	50.9	−13.1	30

plasma bicarbonate from the acid excretion. In four of them bicarbonate had been administered, and the plasma bicarbonate was found much higher than indicated by the urine index. This is usually the case after bicarbonate administration, the urine failing to show the full effect of the alkali on the blood. Of the remaining two cases, one was in coma, and the excretory mechanism was presumably affected. The other case was one of mild acidosis (47.0 per cent plasma CO_2) which gave an index indicating 36.4 per cent, a decidedly more severe condition. Summarized, the results indicate that in diabetic patients short of coma and not receiving bicarbonate the state of the plasma bicarbonate CO_2 can usually be estimated within 10 volumes per cent from the index of acid excretion.

In order to study variations in the same individual, twenty-nine observations were made in the course of a few months upon a patient who entered the hospital with severe acidosis. The result of these repeated tests is shown in Table III.

Of these twenty-nine observations, in five the calculated plasma bicarbonate differed by more than 10 volumes per cent from that found. Three of these discrepancies seemed to depend on the fact that sodium bicarbonate had been given. These observations, like those in Table II, show that sodium bicarbonate disturbs the relationship between blood and urine, the latter failing to show the extent to which the alkali raises the plasma CO_2 capacity.

Use of Curves to Calculate the Index.

In order to simplify the calculation of the index we have prepared a set of curves which enable one by mere inspection to estimate the index with sufficient accuracy, the only data required being (1) the cc. of urine excreted per 24 hours per kilo body weight, and (2) the cc. of 0.1 N NH_3 + titratable acid per liter of urine.

Since D, the rate of excretion per 24 hours, is equal to the product VC (V = volume of urine per 24 hours in liters) the expression $\sqrt{\frac{D}{W}} \sqrt{C}$ may be written $\sqrt{\frac{VC}{W}} \sqrt{C} = \left(\frac{V}{W}\right)^{\frac{1}{2}} C^{\frac{1}{2}}$. Consequently one can plot on coordinate paper the values of the index corresponding to different values of $\frac{V}{W}$ and C.

The accompanying figure indicates the manner in which it appeared simplest to do this.

To use an example: If in a given case the body weight is 50 kg., the volume of urine per 24 hours 2,000 cc., and the NH_3 + titratable acid 1,200 cc. per liter, we have the two values $\frac{D}{W} = \frac{2,000}{50} = 40$ cc. of urine per 24 hours per kg., and $C = 1,200$. We run down the curve corresponding to $C = 1,200$ until it is crossed by the vertical line corresponding to $\frac{V}{W} = 40$, and read off 41 as the value of the index. If the value of C were 1,250, we should estimate one-half of the distance between the 1,200 and 1,300 curves of C, and read 42 as the value of the index.

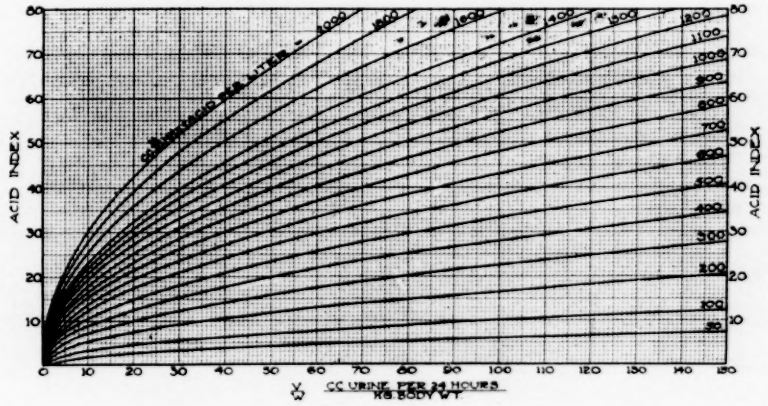

FIG. 1.

SUMMARY.

In normal men and diabetics the excretion of acid in excess of fixed bases as measured by determining ammonia plus titratable acid bears a quantitative relationship to the alkaline reserve of the body as measured by the CO_2 binding power of the blood plasma.

Such relationship is demonstrated by the use of an empirical expression of the form devised by Ambard to denote the relationship between blood concentration and excretion in the cases of salt and urea. The formula used is $\sqrt{\frac{D}{W}}\sqrt{C}$, D representing the rate of excretion of 0.1 N ammonia + titratable acid per 24

hour time unit, C the 0.1 N NH_3 + acid per liter of urine, and W the body weight. The value $80 - \sqrt{\frac{D}{W}}\sqrt{C}$ determined in the urine indicates with an error which is usually less than 10 volumes per cent, the level of the plasma CO_2 capacity. Diabetics receiving bicarbonate administrations are exceptions, the blood bicarbonate in such cases being, as a rule, much higher than indicated by the urine. One case in coma showed a much lower blood bicarbonate than was indicated by the urine.

The index can be determined from analysis of the urine passed in 24 hours or from the amount excreted in 1 or 2 hours, multiplied to bring the data to a 24 hour basis. The calculation is simplified by the use of curves, which make it possible to estimate the index from inspection, the two necessary figures being the cc. of urine passed per kilo body weight per 24 hours, and the amount of ammonia plus titratable acid per liter of the urine.

STUDIES OF ACIDOSIS.

V. ALVEOLAR CARBON DIOXIDE AND PLASMA BICARBONATE IN NORMAL MEN DURING DIGESTIVE REST AND ACTIVITY.

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Higgins[1] and Erdt[2] have both found that digestive activity causes a rise in alveolar carbon dioxide tension. Their explanations of this phenomenon are different. Erdt believed that the change was due to an increase in the reserve alkali of the blood, caused by secretion of hydrochloric acid in the gastric juice. Higgins, however, had in mind the readiness with which slight changes in conditions can affect the alveolar carbon dioxide, without any conceivable effect on blood alkali. For example, he found that changing the posture from standing to lying increased the alveolar carbon dioxide tension by 6 mm., apparently by rendering the respiratory center less irritable. He believed that the effect of a meal was due to a similar influence on respiration, rather than on the alkaline reserve.

It seemed that clarifying evidence might be gained by determining both alveolar carbon dioxide tension and the plasma bicarbonate (under definite carbon dioxide tension as described in Paper I), before and after eating. If the respiratory center remains normal the plasma bicarbonate must rise with the alveolar carbon dioxide, and the ratio $\frac{\text{plasma } CO_2}{\text{alveolar } CO_2}$ remain constant. If, however, the irritability of the center falls, the ratio should fall after meals, the alveolar carbon dioxide increasing,

[1] Higgins, H. L., *Am. J. Physiol.*, 1914, xxiv, 114.

[2] Erdt, H., *Deutsch. Arch. klin. Med.*, 1915, xlvii, 497.

the plasma carbon dioxide either remaining constant, or increasing less in proportion than the alveolar.

Partly to throw light on this question, and partly to obtain statistics on the normal range of plasma bicarbonate values, we have performed these determinations on a number of obliging colleagues. In some cases breakfast, a mixed diet meal taken at about 8 o'clock, was the object of the experiment; in other cases lunch, taken from 4 to 5 hours later. The determinations were made within a half hour before the meal in each case, and between 30 and 60 minutes after it. The blood samples were drawn immediately after the alveolar air samples were taken. The alveolar carbon dioxide samples were taken standing, the Fridericia[3] apparatus being used.

The results are tabulated below.

TABLE I.

Observations before and after Breakfast.

Subject.	Time (before and after meal).	CO_2 bound as bicarbonate by 100 cc. of plasma.	Alveolar CO_2.	$\frac{\text{Plasma } CO_2}{\text{Alveolar } CO_2}$
		cc.	*mm.*	
P.	Before.	68.7	38.7	1.78
	After.	73.5	40.9	1.80
S.	Before.	66.9	41.7	1.60
	After.	63.5	43.9	1.45
M.	Before.	65.4	39.5	1.66
	After.	65.4	43.1	1.52
F.	Before.	68.2	42.8	1.59
	After.	69.1	43.1	1.60

[3] Fridericia, L. S., *Berl. klin. Woch.*, 1914, li, 1268.

TABLE II.

Observations before and after Midday Meal.

Subject.	Time (before and after meal).		CO_2 bound as bicarbonate by 100 cc. of plasma.	Alveolar CO_2.	Plasma CO_2 / Alveolar CO_2
			cc.	*mm.*	
McL. I		Before.	53.4	40.6	1.31
		After.	56.1	49.3	1.27
" II		Before.	56.5	41.6	1.36
		After.	61.0	47.3	1.29
" III		Before.	59.1	40.3	1.47
		After.	61.1	41.7	1.47
P.		Before.	65.1	37.8	1.72
		After.	67.2	40.2	1.68
V. I		Before.	75.0	46.3	1.62
		After.	70.8	47.2	1.50
" II		Before.	72.8	44.0	1.66
		After.	69.8	43.9	1.59
D.		Before.	63.9	45.7	1.40
		After.	68.1	49.8	1.37
S.	1 hr.	Before.	68.4	44.0	1.55
	0.5 "	After.	63.0	46.1	1.37
	1.5 "	"	66.2	48.6	1.36
	2.5 "	"	68.6	47.3	1.45
C.	1 hr.	Before.	68.2	46.2	1.48
	0.5 "	After.	70.7	48.0	1.47
	2.5 "	"	70.7	45.1	1.57

DISCUSSION OF RESULTS.

Normal Range of Plasma Carbon Dioxide Capacity.—This series, together with the thirty analyses performed with the same method by Gettler and Baker,[4] may be taken as establishing the range of plasma carbon dioxide capacity in normal adults.

[4] Gettler, A. O., and Baker, W., *J. Biol. Chem.*, 1916, xxv, 211.

All of our results fall between 53 and 75 cc. of carbon dioxide bound as bicarbonate by 100 cc. of plasma, and all of Gettler and Baker's are between 56 and 78. Consequently the extreme range appears to lie between 53 and 78. Only one plasma out of the fifty-nine failed to bind more than 55 per cent of its volume of CO_2 (McL. I binding 53.4), so that 55 may be taken as the usual minimum.

Normal Relationship between Plasma Bicarbonate and Alveolar Carbon Dioxide.—The ratio $\frac{\text{plasma } CO_2}{\text{mm. alveolar } CO_2}$ varies from 1.27 to 1.80. It shows some tendency toward characteristic levels in given individuals, McL. always showing a low ratio, P. a high one. The average is approximately 1.5. In order to obtain comparison of the alveolar and plasma data, therefore, the alveolar figure may be multiplied by 1.5. Or the plasma figure may be multiplied by $\frac{1}{1.5} = 0.66$ to make it comparable with the alveolar. In making such comparisons, the above variations in the normal ratio must not be forgotten.

Effects of Digestion.—The observation of Higgins and of Erdt is confirmed, that the alveolar carbon dioxide tension rises after a meal. In the one individual in whom a fall was noted (V.), the alveolar carbon dioxide was extremely high in the morning before lunch, probably because of an alkaline vegetarian breakfast.

The plasma bicarbonate in some cases increases slightly, in others does not.

The ratio $\frac{\text{plasma } CO_2}{\text{alveolar } CO_2}$ in four experiments out of thirteen is changed by less than 0.02, which is within the limit of error. In the other nine experiments the ratio is definitely increased. The results therefore favor Higgins' rather than Erdt's explanation as to the chief factor causing increase in alveolar carbon dioxide during digestion, but the nature of the results is such that data on a statistical scale are necessary for a definite decision.

STUDIES OF ACIDOSIS.

VI. THE BLOOD, URINE, AND ALVEOLAR AIR IN DIABETIC ACIDOSIS.

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The work in the present paper constitutes a study of the quantitative measures of acidosis in the blood, urine, and alveolar air of diabetic patients. It has had for its object the accumulation of data sufficient to show: (1) The relationship of plasma bicarbonate deficiency in man to the clinical severity of acidosis; and (2) the relationship of the blood bicarbonate determined directly in the plasma to that estimated indirectly from the excretions of the lungs and kidneys.

The alveolar carbon dioxide tension, under ideal normal conditions, indicates the level of the arterial blood bicarbonate. It has been shown by several authors,[1] however, that the alveolar carbon dioxide tension is readily altered by numerous factors, psychic, pathological, and physiological, besides the blood bicarbonate, and consequently is a reliable measure of the latter only when it is certain that both the mechanical and nervous factors controlling respiration are normal. The alveolar carbon dioxide tension has found its chief clinical application probably in the detection of diabetic acidosis; but so far as we can ascertain no attempt has yet been made to determine whether the quantitative relationship between blood bicarbonate and alveolar carbon dioxide in diabetes is normal or otherwise.

In Paper V we have determined the normal ratio between plasma bicarbonate and alveolar carbon dioxide. In the present paper we have determined the same ratio in twenty-one diabetic

[1] For discussion of the relationship between alveolar carbon dioxide tension and the arterial blood bicarbonate, see Paper I.

patients and one nephritic patient, examined frequently over periods usually of several weeks. We found that in a third of these patients the alveolar carbon dioxide either indicated a bicarbonate deficit when none existed, or indicated a much greater deficit than actually existed. The reverse, *i.e.*, alveolar air too high, was encountered only in the nephritic. The most ready explanation is that the diabetic patients, most of whom were on a low diet, had abnormally irritable respiratory centers.

In Paper IV it has been shown that in most diabetics a quantitative relationship can be demonstrated between the blood plasma bicarbonate and the excretion of ammonia plus titratable acid in the urine. The excretion is expressed by the index $\sqrt{\frac{D}{W}}\sqrt{C}$ (where D represents the rate of excretion of N/10 ammonia plus acid per 24 hours time unit, W the body weight, and C the N/10 ammonia plus acid per liter). The plasma bicarbonate carbon dioxide falls in proportion as the index rises, the relationship being expressed by the equation:

$$\text{Volume per cent plasma } CO_2 = 80 - \sqrt{\frac{D}{W}}\sqrt{C}$$

Excepting cases treated with bicarbonate, the plasma carbon dioxide in acidosis could, as a rule, be estimated from the urine to within ± 10 volume per cent. It was desirable to obtain further data concerning the regularity of this relationship between blood and urine in diabetes by systematic observations over considerable periods of time on patients in varying stages of acidosis. On sixteen of the twenty-two patients studied we have consequently followed the acid index, together with the other determinations, throughout the periods of observation. Reference to the charts shows that on the whole the urine index of acid excretion, considering its purely empirical nature, agrees surprisingly well with the plasma bicarbonate.

As additional controls on the blood itself, the acid-neutralizing power of the plasma was estimated by the electrometric titration (Paper III) and the carbon dioxide capacity of the whole blood as well as of the plasma was determined. The electrometric titration was performed as a measure of the *total* buffer content of the plasma. The object in determining the carbon

dioxide capacity of the whole blood was to ascertain whether the technique employed in plasma analyses occasioned any errors, caused by loss of carbon dioxide from the blood and consequent effect on the acid-base transfer between plasma and corpuscles (Paper I).

Methods.

Blood samples were drawn from the arm vein into a McRae tube as described in Paper I. A portion of each sample was used at once for determination of the carbon dioxide binding power of the whole blood, and while this determination was being performed the remainder of the blood was centrifuged, the plasma so obtained being used for determining the carbon dioxide binding power and for the electrometric titration.

The alveolar carbon dioxide tension was determined by Fridericia's[2] modification of the Haldane method. Determinations were, as a rule, repeated until duplicates were obtained agreeing within 0.1 per cent of carbon dioxide. The method was repeatedly checked by determinations on normal individuals. The patients were well trained in their part of the technique and cooperated satisfactorily. We were surprised at the low results given by some patients with normal or but slightly subnormal plasma carbon dioxide capacity, and tested our technique as completely as possible in order to find a source of error, but were able to discover none.

The electrometric titration of the plasma was determined as described in Paper III.

The carbon dioxide bound as bicarbonate by the plasma was determined as described in Paper I.

The carbon dioxide bound as bicarbonate by the whole blood at normal alveolar temperature and tension was determined as follows: Several cc. of freshly drawn blood were placed in a 300 cc. separatory funnel and warmed to 37°C. (thermometer in funnel) in an incubator. The funnel was then filled with an artificial air-carbon-dioxide mixture containing 5.5 per cent of carbon dioxide, and the blood was saturated with this gas by rotating the funnel. The time required to saturate is longer

[2] Fridericia, L. S., *Berl. klin. Woch.*, 1914, li, 1268.

than the 2 minutes which suffice for plasma, and in order to insure its completeness the second duplicate was usually done after repeating the saturation. The carbon dioxide determinations were made as described in Paper II. A correction of 3 volume per cent was subtracted for carbon dioxide physically dissolved by the blood under the conditions of saturation.

The index of acid excretion in the urine was determined as described in Paper IV. Aliquot parts of the urine passed during the whole 24 hour period were used for the determinations of ammonia and titratable acid.

Urea was determined in the urine, as a measure of the nitrogen metabolism and concentration of normal urinary constituents, by Marshall's urease method as modified by Van Slyke and Cullen.[3]

Explanation of Charts.

In the cases where all five determinations were performed, the five sets of results obtained are graphically represented by two sets of curves, the blood analyses in one set, on the upper half of the charts, and the urine and alveolar air in another on the lower half. In order to facilitate comparison of the latter with plasma carbon dioxide, its curve is reproduced in the lower as well as in the upper set.

In both sets of curves a straight line is drawn across at the level indicating the minimum normal carbon dioxide capacity, 55, of adult human plasma. When the plasma curve drops below this line, a condition of acidosis is indicated. The curves of the *upper* set should normally run parallel, but not coincide. The capacity of whole blood to combine with 5.5 per cent carbon dioxide at 37° is about 15 volume per cent less than that of plasma at 20°. Consequently the whole blood curve, where there is no acidosis, may be expected to run parallel to the plasma curve, but below it by a difference indicating about 15 volume per cent carbon dioxide. As acidosis develops and both curves sink toward zero, this difference, of course, diminishes. With these relationships in mind, one may use the whole blood capacity to check the plasma capacity for irregular drops such as could be

[3] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 211; 1916, xxiv, 117.

caused by large losses of carbon dioxide from the blood sample, and consequent transfer of HCl from corpuscle to plasma, before the blood is centrifuged (Paper I). Such a drop undoubtedly occurred in one plasma determination (Chart 5, Case 2686, March 3), but apparently in only that one out of the scores that were thus controlled.

The electrometric titration curves are so arranged that the normal and extreme acidosis values approximately coincide with those of the plasma CO_2 curves, but the natures of the determinations are so different that only an approximate parallelism can be expected. This is shown quite consistently.

The curves of the *lower* sets are so arranged that if alveolar carbon dioxide and urine excretion maintain normal relationships with the plasma bicarbonate, all three curves should coincide. The alveolar carbon dioxide tension expressed in mm. is multiplied by 1.45 in order to make it comparable to the plasma carbon dioxide capacity expressed in volume per cent. The ratio in normal men varies from 1.3 to 1.8 (Paper V), so that a fairly wide margin for normal deviations must be allowed. But when the alveolar $CO_2 \times 1.45$ runs over 10 units below the plasma curve the deviation must be taken as abnormal.

The urine index curves also are so plotted that absolute agreement with the plasma results would be indicated by the exact coincidence of the two curves. The degree of deviation in any given case is a measure of the error that would be made in estimating the alkaline reserve of the blood from the acid excretion.

In tabulating the diets, the caloric values when only green vegetables were fed were omitted, because the digestible protein and fat are so uncertain as to make the caloric value indefinite (approximately 6 to 7 calories for each gm. of carbohydrate in green vegetable ingested). When whiskey was given during these periods the caloric value of the alcohol is tabulated.

To the note accompanying each chart is appended the group to which the case belongs according to Stillman, who has classified diabetics as follows:[4]

Group I is composed of cases which maintain a normal bicarbonate reserve of the blood throughout the course of their treatment.

[4] Stillman, E., *Am. J. Med. Sc.*, 1916, cli, 505.

SUMMARY OF CHARTS AND TABLES.

No.	Case No.	Condition.	Observation period.	Acid excretion index of urine compared with plasma CO_2.	Alveolar CO_2 compared with plasma CO_2.
			days		
1	2481	Diabetic with pronounced acidosis during part of period.	31	Not determined.	+*
2	2646	"	22	+*	Alveolar low.
3	2620	"	20	+	" "
4	2680	"	19	+	" "
5	2686	"	22	+	+
6	2414 (1916)	"	25	+	Alveolar low.
7	2414 (1915)	"	49	Not determined.	+
8	2293	"**	4	" "	+
9	2358	"	35	" "	+
10	2684 Feb., 1916	"	21	+	Alveolar low.
11	2684 Jan., 1916	"	23	+	Not determined.
12	2128 (1916)	"	11	+	" "
13	2382	Diabetic with mild but definite acidosis.	47	Not determined.	+
14	2332	"	36	" "	+
15	2343	"	36	" "	+
16	2128 (1915)	"	33	" "	+
17	2480	Diabetic without acidosis.	19	+	+
18	2234	"	14	+	Alveolar low.
19	2374	"	39	Not determined.	+
20	2389	"	33	" "	+
21	2394	"	34	" "	+
22	2366	Nephritic with acidosis.	61	Not determined.	Alveolar 15 mm. too high.

*The + sign indicates approximate agreement of urine or alveolar air with plasma carbon dioxide.

**Fatal.

In Group II are placed the cases which recover, while fasting, from acid intoxication, sometimes so severe as to verge on coma.

Group III consists of those cases which show a low grade acidosis as indicated by a subnormal bicarbonate reserve and heightened ammonia excretion.

Group IV includes those cases which develop acidosis while fasting, though previously no evidence of acid intoxication existed.

Reference to the charts and tables, and summarizing the general results expressed by them, will be facilitated by use of the table on page 410.

CONCLUSIONS.

The consistent agreement of the carbon dioxide capacity of the plasma and whole blood respectively indicate that, with the technique used for handling blood samples, no significant error is caused by the acid-base transfer between corpuscle and plasma in the period between the drawing and centrifuging of the blood.

The electrometric titration of the plasma roughly parallels the carbon dioxide combining capacity, the agreement showing that the total buffer content of the plasma, determined by the electrometric titration, is approximately proportional to the bicarbonate content.

The alveolar carbon dioxide tension in diabetic patients under treatment is often much too low to indicate the true level of the blood bicarbonate. The alveolar carbon dioxide tension has been observed as low as 26 mm. with a normal plasma bicarbonate. Error in the opposite direction, an alveolar carbon dioxide which is too high, and therefore fails to show an existing acidosis, we have never encountered in diabetes (it is encountered in the one case of nephritis shown in the last chart). When severe diabetic acidosis causes both alveolar carbon dioxide and plasma bicarbonate to fall to a low level, the discrepancy noted above diminishes. When the plasma carbon dioxide has been below 40 volume per cent the agreement between the alveolar and the plasma figures has usually been close.

Excepting those days when bicarbonate was administered, the consistent agreement of the figure for plasma CO_2, calculated as $80 - \sqrt{\frac{D}{W}}\sqrt{C}$ from Fitz' index of acid excretion in the

urine, with the CO_2 determined directly in the plasma is striking. The agreement of urine and blood is on the average more accurate than that of the alveolar air and blood, and is observed not only in adults but also in children of as little as 25 kilos weight.

In very severe acidosis, however, the urine index is less accurate than the alveolar air in indicating the alkaline reserve. With a plasma carbon dioxide of 25 per cent, which corresponds to a urine index of 55, the index may be 65, indicating 15 per cent plasma carbon dioxide, which in our experience is fatal, or it may be 45, indicating 35 per cent plasma carbon dioxide, which though a pronounced acidosis is so well above the danger limit that signs of coma are usually absent.

Of the two indirect measures of alkaline reserve, therefore, the alveolar carbon dioxide appears the more accurate in measuring the more severe stages of diabetic acidosis, such as are encountered in threatened coma, while the index of acid excretion is the more accurate in measuring the more common intermediate stages.

The data published in this and the preceding papers appear sufficiently complete to warrant the generalizations expressed in the following table.

Range of Results Obtained with Normal and Pathological Plasma.

Condition of subject.	Plasma CO_2 capacity.		Corresponding values of acid index of urine $\sqrt{\frac{D}{W}}\sqrt{C}$ Deviations of ± 10 must be allowed for.
	Reading of apparatus. Gas from 1 cc. plasma.	CO_2 reduced to 0°, 760 mm., bound as bicarbonate by 100 cc. plasma.	
	cc.	cc.	
Normal resting adult,* extreme limits.	0.90–0.65	77–53	3–27
Mild acidosis. No visible symptoms	0.65–0.52	53–40	27–40
Moderate acidosis. Symptoms may be apparent	0.52–0.41	40–30	40–50
Severe acidosis. Symptoms of acid intoxication	Below 0.41	Below 30	Over 50
Lowest CO_2 observed with recovery	0.26	16	64

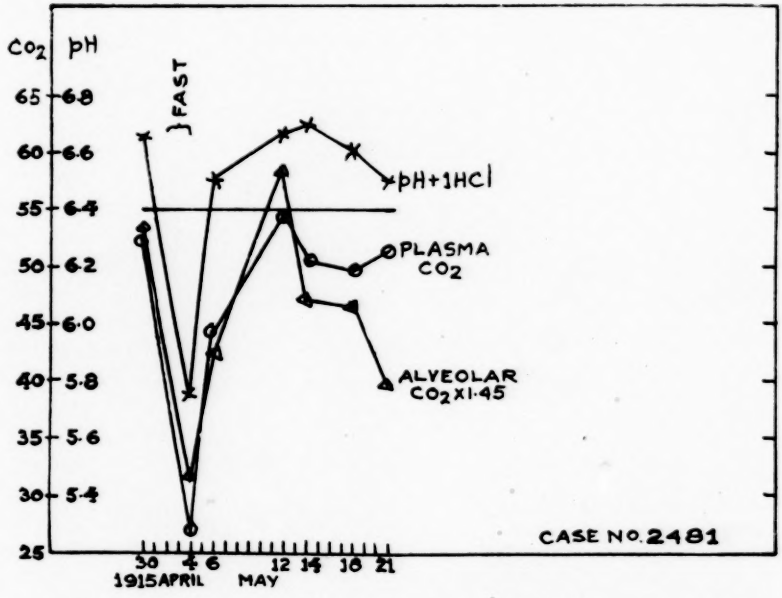
*Schloss (*Am. J. Dis. Child.*, 1917, xiii, 218) finds the carbon dioxide bound by the plasma of normal infants to be 46 to 63 cc. per 100 cc. of plasma, about 10 cc. lower than in adults.

CASES

1. Case 2481.

Date.	Total calories.	Protein.	Fat.	Carbohydrate.	Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio $\frac{\text{plasma } CO_2}{\text{mm. alv. } CO_2}$	Plasma + 1 vol. N/50 HCl.
1915		*gm.*	*gm.*	*gm.*	*mm.*	*mm.×1.45*	*cc.*		*pH*
Apr. 30	80	7.5	5.0		37.0	53.6	52.2	1.41	6.66
May 1	160	15.0	10.0						
2	Fast day.								
3*	" "								
4	Green veg. only containing			16	21.5	31.2	27.0	1.26	5.75
5	" " " "			40.5					
6	" " " "			60.0	29.2	42.3	44.1	1.51	6.51
7†	" " " "			11.5					
8	375	29.0	25.0	5.0					
9	630	50.0	43.0	"					
10	"	"	"	"					
11	"	"	"	"					
12	"	"	"	"	40.2	58.2	54.5	1.35	6.67
13	"	"	"	"					
14	655	51.5	43.5	10.0	32.4	47.0	50.2	1.55	6.70
15	170†	13.5	12.5						
16	Fast day.								
17	Green veg. only containing			10.0					
18	" " " "			20.0	32.0	46.4	51.9	1.62	6.61
19†	" " " "			12.5					
20	Fast day.								
21	355	24.5	23.5	10.0	27.3	39.6	51.2	1.88	6.50

*Drowsiness. †Partial fast.



1. Case 2481, female, age 12. Diabetic less than 3 months, mild, fair ability to burn carbohydrate (shown subsequently by a carbohydrate tolerance test).

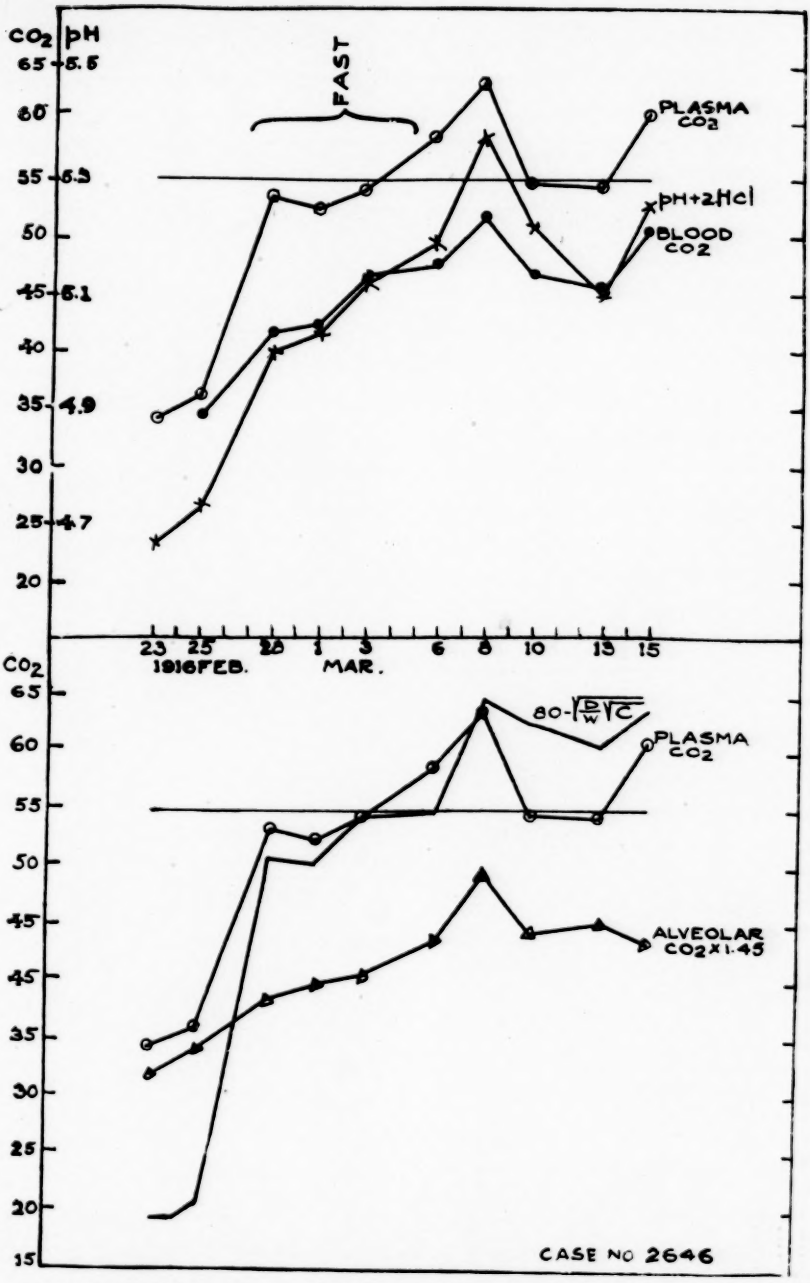
The curves show a dangerous acidosis when the patient was placed on a fast. This was accompanied by clinical symptoms. The acidosis was ameliorated when a carbohydrate diet was instituted. The drop in alveolar CO_2 on the last day was evidently due merely to change in respiratory control.

(From April 30–May 21, 1915.) Group IV.

2. Case 2646.

Date.	Diet.				Body weight.	Blood.						Urine.					
	Total calories.	Protein	Fat.	Carbohydrate.		Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio plasma CO_2 / mm. alv. CO_2	CO_2 bound by 100 cc. whole blood at 37°.	Plasma + 2 vol. N/50 HCl.	Vol. per 24 hrs.	Urea per liter.	NH_3 per liter.	Acid per liter.	$\sqrt{\frac{D}{W}}\sqrt{C}$	$80-\sqrt{\frac{D}{W}}\sqrt{C}$
1916		*gm.*	*gm.*	*gm.*	*kg.*	*mm.*	*mm. × 1.45*	*cc.*		*cc.*	*pH*	*cc*	*cc. N/10*	*cc. N/10*	*cc. N/10*		
Feb.																	
23*	2,000	75	138	100	37	22.2	31.9	34.3	1.54		4.67	2,600	1,650	1,175	224	60.6	19.4
24*	"	"	"	"	37.4							2,640	1,440	1,183	204	60.7	19.3
25*	"	"	127	125	37.6	23.5	34.1	36.0	1.53	34.7	4.73	2,560	1,318	1,205	174	59.5	20.5
26	"	"	"	"													
27	Fast day.																
28	"	"			37.7	26.4	38.3	53.4	2.02	41.9	5.0	2,625	390	456	54	29.1	50.9
29	"	"															
Mar.																	
1	"	"			37.2	27.3	39.6	52.1	1.91	42.1	5.03	1,950	650	568	74	29.7	50.3
2	"	"															
3	"	"			35.6	28.0	40.6	54.0	1.93	46.5	5.12	2,305	530	420	62	26.1	53.9
4	"	"															
5	Green veg. only containing			10													
6	"	"	"	20	35.6	30.0	43.5	58.9	1.97	47.4	5.19	2,140	754	423	64	25.2	54.8
7	"	"	"	"													
8	"	"	"	8.6	36.9	34.1	49.5	63.4	1.86	51.6	5.38	3,045	424	182	22	15.7	64.3
9	80	7.5	5.0														
10	160	15	10.5		36.3	30.5	44.2	54.9	1.80	46.8	5.22	2,600	598	232	28	17.3	62.7
11	320	30	21.0														
12	Fast day.																
13	Green veg. only containing			10.0	36.3	31.2	45.2	54.4	1.74	45.2	5.10	2,470	556	270	32	19.7	60.3
14	"	"	"	20.0													
15	"	"	"	30.0	35.8	29.8	43.2	60.9	2.04	50.9	5.26	3,010	416	164	60	16.5	63.5

*Abdominal cramps.



2. Case 2646, female, age 19. Diabetic of moderate severity for 4 years. There was a marked acidosis on admission (February 19, 1916).

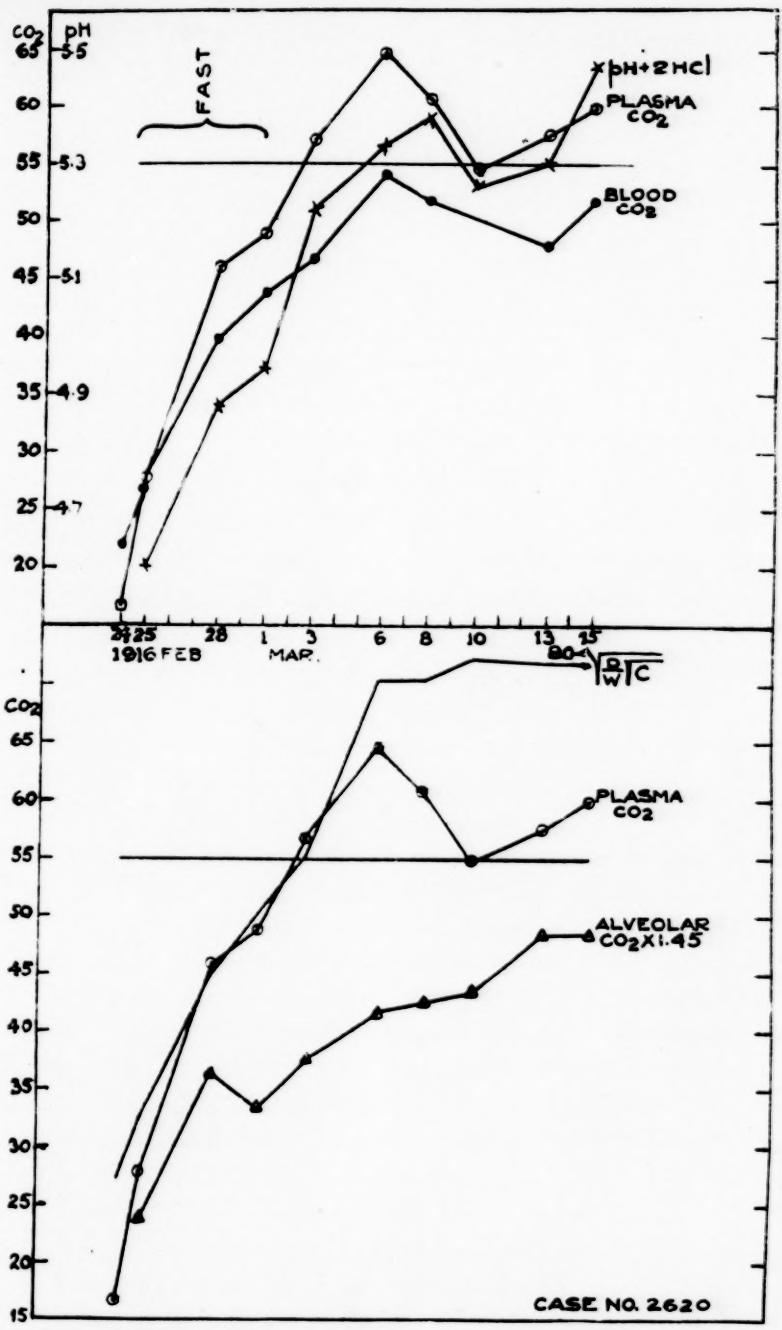
The chart shows the curves, which had remained constant at a level of alarming acidosis for 7 days on a moderate mixed diet containing 100 gm. of carbohydrate, immediately rise to a low normal level on fasting without $NaHCO_3$, gain a high level on a carbohydrate test, regress to a lower level again when a low carbohydrate-free diet was instituted, and again rise to normal when placed on another carbohydrate tolerance test. After recovery from acidosis the alveolar CO_2 is consistently much lower than it should be to indicate the blood bicarbonate.

(From February 23–March 15, 1916.) Group II.

3. Case 2620.

Date.	Diet.				Body weight.	Blood.						Urine.					
	Total calories.	Protein.	Fat.	Carbohydrate.		Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio $\frac{\text{plasma } CO_2}{\text{alv. } CO_2}$	CO_2 bound by 100 cc. whole blood at 37°.	Plasma $+\frac{2}{3}$ vol. HCl.	Vol. per 24 hrs.	Urea per liter.	NH₃ per liter.	Acid per liter.	$\frac{D}{W}\sqrt{C}$	$80-\sqrt{\frac{D}{W}\sqrt{C}}$
1916		*gm.*	*gm.*	*gm.*	*kg.*	*mm*	*mm. × 1.45*	*cc.*		*cc.*	*pH*	*cc.*	*cc. N/10*	*cc. N/10*	*cc. N/10*		
Feb.																	
24*	Fast day.				25			16.6		21.9		1,800	2,110	747	230	52.8	27.2
25	"	"			25.6	16.3	23.6	27.7	1.70	27.5	4.61	2,905	1,165	580	144	47.4	32.9
26	"	"															
27	"	"															
28	"	"			25.2	25.0	36.3	45.9	1.83	39.6	4.88	3,440	692	370	44	35.1	44.9
29	"	"															
Mar.																	
1	Green veg. only containing			10	25.3	23.0	33.4	48.7	2.12	43.8	4.94	3,675	682	304	28	29.8	50.2
2	"	"	"	"	20												
3	"	"	"	"	30	26.4	25.9	37.6	56.9	2.20	46.5	5.22	3,300	532	260	20	24.8	55.2
4	"	"	"	"	40												
5	"	"	"	"	50												
6	"	"	"	"	60	27.0	28.6	41.5	64.6	2.26	54.0	5.33	3,025	656	99	−12	9.7	70.3
7	"	"	"	"	70												
8	"	"	"	"	80	26.4	29.2	42.3	60.7	2.08	51.6	5.38	3,165	750	70	10	9.7	70.3
9	"	"	"	"	90												
10	"	"	"	"	100	25.8	29.8	43.2	54.5	1.83		5.26	3,260	772	57	4	7.9	72.1
11	"	"	"	"	110												
12	"	"	"	"	120												
13	"	"	"	"	130	25.8	33.3	48.3	57.3	1.72	47.5	5.30	2,655	960	55	18	8.1	71.9
14	"	"	"	"	140												
15	"	"	"	"	150	25.8	33.3	48.3	59.9	1.80	51.9	5.48						

*Drowsiness; hyperpnea.



3. Case 2620, male, age 12. Diabetic 1 month, severe in that an extreme acidosis was present, as indicated both by laboratory and physical findings, but mild in carbohydrate tolerance (subsequently proven by a carbohydrate tolerance test).

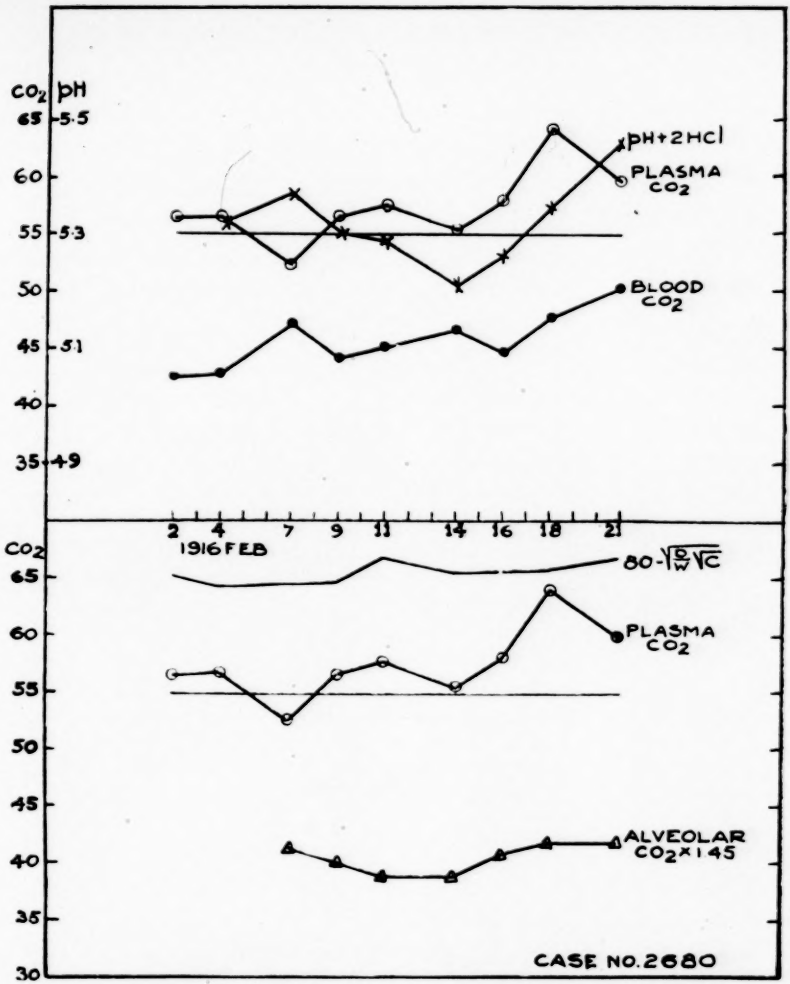
On admission the curves indicated extreme acidosis. 6 days of fasting (without $NaHCO_3$) obviated danger from coma. The subsequent 15 days of his carbohydrate tolerance test brought the acid curves to a normal level, at which they were maintained for the remainder of his stay in the hospital. The alveolar CO_2 after recovery from acidosis was consistently very much lower than the plasma CO_2.

(From February 24–March 15, 1916.) Group II.

4. Case 2680.

Date.	Diet.					Body weight.	Blood.						Urine.					
	Total calories.	Protein.	Fat.	Carbohydrate.	Alcohol.		Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio plasma CO_2 / mm. alv. CO_2	CO_2 bound by 100 cc. whole blood at 37°.	Plasma $+\frac{2}{3}$ vol. $\frac{N}{50}$ HCl.	Vol. per 24 hrs.	Urea per liter.	NH_3 per liter.	Acid per liter.	$\frac{D}{W\sqrt{C}}$	$80-\frac{D}{W\sqrt{C}}$
1916		*gm.*	*gm.*	*gm.*	*cc.*	*kg.*	*mm.*	*mm. × 1.45*	*cc.*		*cc.*	*pH*	*cc.*	*cc. N/10*	*cc. N/10*	*cc. N/10*		
Feb.																		
2	765	22.5	16	0	75	38.0			56.3		42.3		2,855	792	138	63	14.9	65.1
3	805	32.5	"	"	"													
4	"	"	"	"	"	37.4			56.4		42.6	5.32	2,540	1,465	190	54	15.9	64.1
5	"	"	"	"	"													
6	525	Fast day.																
7	650	32.5	16		53	37.2	28.4	41.2	57.3	2.02	47.0	5.37	2,015	1,690	195	84	15.6	64.4
8	"	"	"	0	"													
9	625	25.0	"	"	"	38.0	27.6	40.0	56.6	2.05	44.0	5.30	2,065	1,190	202	74	15.5	64.5
10	450*	7.5	5.5	"	"													
11	370	Fast day.				37.7	26.8	38.9	67.8	2.16	45.0	5.29	2,425	1,285	147	46	13.1	66.9
12	450*	7.5	5.5	0	53													
13	505	"	9.5	"	"													
14	530	15.0	10.5	"	"	38.0	26.8	38.9	55.2	2.06	46.6	5.21	2,710	925	158	52	14.7	65.3
15	610	22.5	16.0	"	"													
16	440	15.0	10.5	"	40	37.9	28.1	40.8	58.0	2.06	44.5	5.26	2,380	1,250	214	58	16.7	63.3
17	"	"	"	"	"													
18	320	4.0	2.5	"	"	37.6	28.8	41.8	64.1	2.22	47.9	5.35	2,730	925	167	36	14.4	65.6
19	360*	7.5	5.5	"	"													
20	" *	"	"	"	"													
21	400	11.0	8.0	"	"	37.2	28.8	41.8	59.9	2.08	50.1	5.46	2,865	902	147	34	13.3	66.4

* Partial fast.



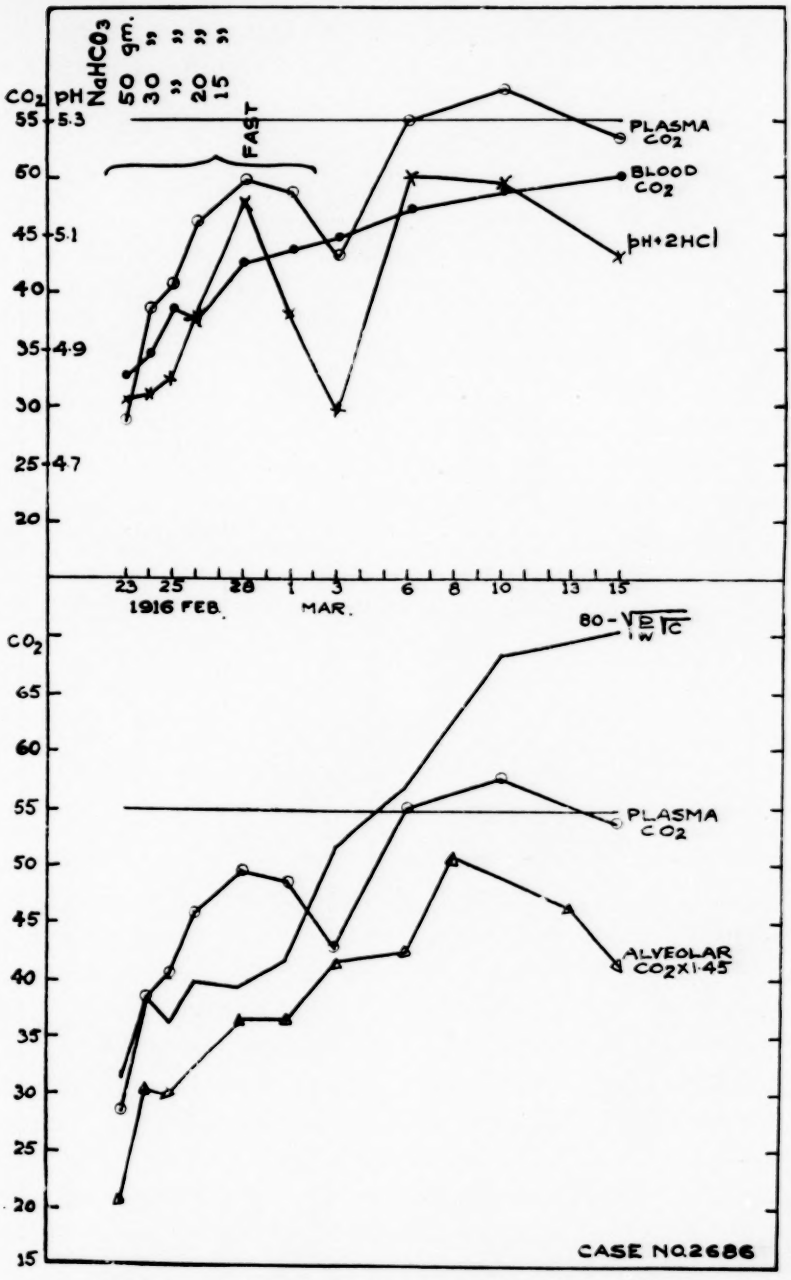
4. Case 2680, female, age 29. Diabetic 10 months, of the extremest type, as shown by the inability to tolerate the 1,000 calories allowed although nearly half of the caloric intake was composed of alcohol. A moderate acidosis was manifested on admission (October 16, 1915).

The curves (taken during the 4th month of hospital observation) show a tendency to acidosis by continually approaching the lower border of normal. Possibly this is the result of the necessarily low diet. The alveolar CO_2 is much too low to show the true blood bicarbonate.
(From February 2–21, 1916.) Group III.

5. Case 2686.

Date.	Diet.					Body weight.	Blood.						Urine.					
	Total calories.	Protein.	Fat.	Carbohydrate.	$NaHCO_3$.		Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio $\frac{\text{plasma } CO_2}{\text{mm. alv. } CO_2}$	CO_2 bound by 100 cc. whole blood at 37°.	Plasma $+\frac{2}{\text{vol. HCl}}$ s/50	Vol. per 24 hrs.	Urea per liter.	NH_3 per liter.	Acid per liter.	$\frac{D}{W}\sqrt{C}$	$80-\sqrt{\frac{D}{W}\sqrt{C}}$
1916	*gm.*			*gm.*	*gm.*	*kg.*	*mm.*	*mm.×1.45*	*cc.*		*cc.*	*pH*	*cc.*	*cc. N/10*	*cc. N/10*	*cc. N/10*		
Feb.																		
22*	Fast day.				45													
23**	"	"			50	27.8	14.0	20.3	28.6	2.04	32.7	4.81	6,530	915	310	128	48.7	31.3
24†	"	"			30	26.0	20.8	30.2	38.3	1.84	33.5	4.82	4,915	732	317	128	42.6	38.4
25†	"	"			30	25.4	20.6	29.9	40.4	1.96	38.3	4.85	4,760	718	362	112	43.9	36.1
26	"	"			20	25.0			46.0		37.8	4.97	3,115	888	481	92	40.1	39.9
27	"	"			15													
28	"	"				25.4	25.3	36.7	49.6	1.96	42.8	5.16	3,335	575	483	60	40.7	39.3
29	"	"																
Mar.																		
1	"	"				25.0	25.2	36.5	48.7	1.93	43.8	4.96	3,685	482	336	36	38.5	41.5
2	Green veg. only containing				10													
3	"	"	"	"	20	25.6	20.88	41.8	43.0	1.49	44.7	4.79	1,635	862	526	38	28.3	51.7
4	"	"	"	"	"													
5	"	"	"	"	25													
6	"	"	"	"	"	30.0	29.2	42.3	55.0	1.89	47.0	5.20	2,330	464	342	20	23.1	56.9
7	"	"	"	"	"													
8	"	"	"	"	30	31.8	28.5						2,965	436	185	14	16.5	63.5
9	"	"	"	"	35													
10‡	"	"	"	"	8.8	31.6	35.5	50.8	57.6	1.62	48.5	5.19	2,820	502	113	60	11.7	68.3
11	Fast day.																	
12	Green veg. only containing				5													
13	"	"	"	"	10	29.8	31.9	46.3					3,145	474	116	18	9.8	70.2
14	"	"	"	"	20													
15	"	"	"	"	"	29.4	28.4	41.2	53.6	1.88	50.0	5.06	3,520	332	69			

* Drowsiness; hyperpnea; nausea; practically moribund. ** Drowsiness; hyperpnea. † Hyperpnea. ‡ Partial fast.



5. Case 2686, male, age 13. Diabetic 1 year, extreme in acid intoxication, rather severe in carbohydrate tolerance (subsequently shown by a carbohydrate tolerance test).

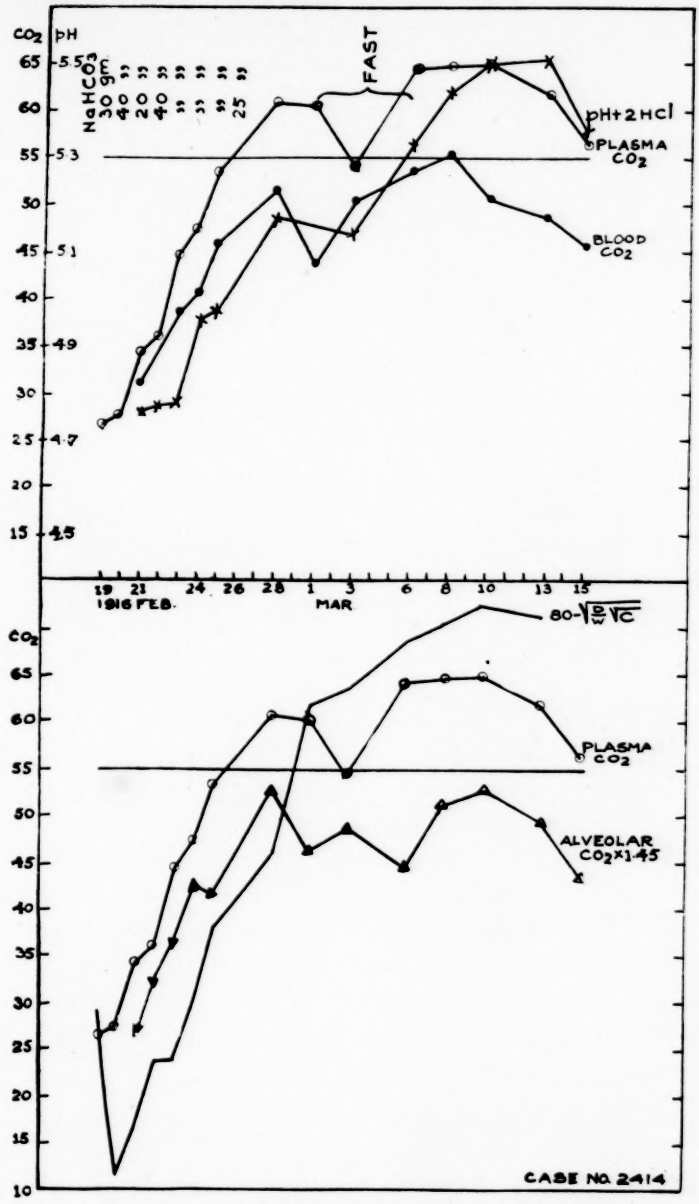
The curves indicate an acidosis bordering on coma, rapidly coming out of danger through fasting with $NaHCO_3$ therapy, but maintaining a subnormal level throughout. It might be prognosed from these curves, in such a young case, that the course of the disease, even under a strict régime, would be progressive. The alveolar CO_2 is consistently lower than the plasma bicarbonate. On March 3 the plasma bicarbonate and hydrogen ion concentration show a fall which is presumably due to exposure of the blood sample to CO_2 before centrifugation, since the whole blood figure as well as the acid excretion and alveolar CO_2 show no drop on this date. The absence of this phenomenon in the curves of other cases indicates the rarity of this accident.

(From February 22–March 15, 1916.) Group II–III.

6. Case 2414.

Date.	Diet.					Body weight.	Blood.						Urine.					
	Total calories.	Protein.	Fat.	Carbohydrate.	$NaHCO_3$		Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio plasma CO_2 mm. alv. CO_2	CO_2 bound by 100 cc. whole blood at 37°.	Plasma $+\frac{2}{N/50}$ vol. HCl.	Vol. per 24 hrs.	Urea per liter.	NH_3 per liter.	Acid per liter.	$\sqrt{\frac{D}{W}}\sqrt{C}$	$80-\sqrt{\frac{D}{W}}\sqrt{C}$
1916		*gm.*	*gm.*	*gm.*	*gm.*	*kg.*	*mm.*	*mm.×1.45*	*cc.*		*cc.*	*pH*	*cc.*	*cc. N/10*	*cc. N/10*	*cc. N/10*		
Feb.																		
19*	845	162.5	19	0	30	44.6			26.4				1,130	3,470	1,110	544	50.8	29.2
20	600	94.	23	"	40	44.9			24.2				3,775	3,480	1,102	352	68.56	11.4
21	"	"	"	"	20	44.8	18.0	26.1	34.3	1.90	31.0	4.86	3,980	4,010	1,025	252	63.6	16.4
22	"	"	"	"	40	44.6	22.3	32.2	36.0	1.61		4.87	3,620	3,885	1,065	264	61.4	18.6
23	400	63.	15	"	"	44.6	25.0	36.3	44.5	1.78	38.3	4.88	4,005	3,910	955	248	61.4	18.6
24	"	"	"	"	"	45.0	29.4	42.6	47.2	1.61	40.4	4.96	3,395	3,685	833	208	55.0	29.5
25	"	"	"	"	"	46.0	28.4	41.2	53.3	1.88	45.6	4.98	3,375	3,040	624	200	42.0	38.0
26	200	31.5	7.5	"	25				65.5									
27	"	"	"	"														
28	"	"	"	"		48.9	36.4	52.8	60.9	1.67	51.3	5.18	5,610	2,080	339	122	34.1	45.9
Mar.																		
1	Fast day.					48.0	31.7	46.0	60.2	1.90	43.3		5,080	1,240	175	60	19.3	60.7
2	" "																	
3	" "					47.4	33.8	48.9	54.0	1.60	50.3	5.14	3,450	1,235	179	60	16.9	63.1
4	" "																	
5	" "																	
6	Green veg. only containing			10		47.0	37.0	44.5	64.1	1.73	53.1	5.33	3,290	1,060	117	32	11.4	68.6
7	" " " "			10														
8	" " " "			15		47.2	35.5	51.5	64.4	1.81	55.1	5.44	3,500	1,140	100	18	9.7	70.3
9	" " " "			20														
10	" " " "			25		46.2	35.5	51.5	64.8	1.82	50.5	5.50	3,175	895	830	8	7.6	72.4
11	" " " "			30														
12	" " " "			3.3														
13	160	15	10	0		48.4	34.1	49.5	61.7	1.81	48.9	5.51	3,315	1,095	78	24	8.8	71.2
14	240	22.5	16	0														
15	Green veg. only containing			10.0		44.4	29.8	43.2	56.2	1.89	45.2	5.34	3,275	1,042	97	24	10.0	70.0

* Drowsiness; hyperpnea; nausea.



6. Case 2414, male, age 17. Diabetic 1 year of a rapidly progressive type. Extreme acidosis on his first admission (March 19, 1915), responded to the fasting treatment, and again on the present admission. On this occasion the acidosis was precipitated by a self-directed fast of 7 days.

The chart shows the curves, in a patient brought to the border of coma by ill-advised fasting, respond to treatment consisting of a low protein-fat (carbohydrate-free) diet, sodium bicarbonate in moderate dosage, and forcing of fluid. After recovery from this extreme acidosis, the alveolar CO_2 remained consistently subnormal. The acid excretion during $NaHCO_3$ feeding is, as usual, greater than corresponds to the degree of internal acidosis. The discrepancy disappears the day after $NaHCO_3$ feeding ended.

(From February 19–March 15, 1916.) Group IV.

7. Case 2414.

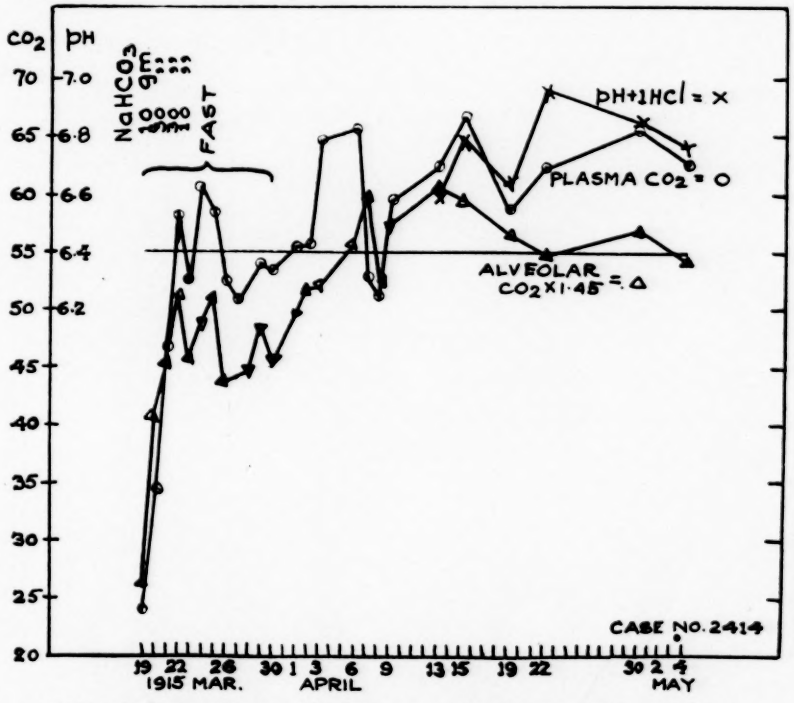
Date.	Total calories.	Protein.	Fat.	Carbohydrate.	$NaHCO_3$.	Alcohol.	Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio plasma CO_2 / mm. alv. CO_2.	Plasma + 1 vol. n/50 HCl.
1915		*gm.*	*gm.*	*gm.*	*gm.*	*cc.*	*mm.*	*mm. × 1.45*	*cc.*		*pH*
Mar.											
19*	245	Fast day with whiskey.			10	35	17.9	26.0	23.8	1.33	
20†	560	"	"	"	30	80	28.0	40.6	34.1	1.22	
21	"	"	"	"	30	80	31.5	45.7	46.8	1.49	
22	525	"	"	"	10	75	35.2	51.1	58.5	1.66	
23	560	"	"	"		80	31.5	45.7	52.4	1.66	
24	385	"	"	"		55	33.7	48.9	60.6	1.80	
25		"	"				35.4	51.4	58.0	1.64	
26		"	"				30.2	43.8	52.4	1.73	
27		"	"						50.4		
28		"	"				30.6	44.4			
29		"	"				33.3	48.3	54.0	1.62	
30	Green veg. only containing			10			31.1	45.1	53.4	1.72	
31	"	"	"	"	"						
Apr.											
1	"	"	"	"	20		34.2	49.6	55.2	1.62	
2	"	"	"	"	"		35.9	52.0	55.2	1.54	
3	"	"	"	"	30		35.9	52.0	64.8	1.80	
4	"	"	"	"	50						
5	"	"	"	"	70						
6	"	"	"	"	80		38.5	55.8	65.6	1.70	
7	"	"	"	"	90		41.4	60.0	52.9	1.28	
8	"	"	"	"	100		35.4	51.4	51.4	1.45	
9	"	"	"	"	150		39.4	57.2	59.5	1.51	
10	"	"	"	"	"						
11	"	"	"	"	"						
12	"	"	"	"	175						
13	"	"	"	"	"		41.7	60.5	62.1	1.49	6.60
14	"	"	"	"	"						
15	"	"	"	"	200		41.0	59.5	66.6	1.62	6.80
16	Fast day.										
17	765	33	56	25							
18	1,240	48	100	"							
19	1,670	"	147.5	"			38.7	56.1	58.5	1.51	6.63
20	"	"	"	"							
21	"	"	"	"							
22	"	"	"	"			37.8	54.8	62.1	1.64	6.97
23	"	"	"	"							
24	1,700	50	149.5	"							

* Drowsiness; hyperpnea.

† Drowsiness.

7. Case 2414—Concluded.

Date.	Total calories.	Protein.	Fat.	Carbohydrate.	$NaHCO_3$.	Alcohol.	Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio plasma CO_2 / mm. alv. CO_2.	Plasma + 1 vol. N/8.50 HCl.
1915		*gm.*	*gm.*	*gm.*	*gm.*	*cc.*	*mm.*	*mm. × 1.45*	*cc.*		*pH*
Apr. 25	Fast day.										
26	1,835	53	156.5	40							
27	1,845	55	"	"							
28	"	"	"	"							
29	"	"	"	"							
30	1,840	52.5	"	"			39.3	57.0	65.6	1.67	6.86
May 1	"	"	"	"							
2	Fast day.										
3	1,840	52.5	156.5	40							
4	1,985	75.5	162.5	"			37.2	54.0	62.3	1.67	6.77



7. Case 2414, male, age 16. Diabetic less than 1 month. Mild in tolerance for carbohydrate (proven by subsequent carbohydrate tolerance test) but extreme in acid intoxication, the patient being on the verge of coma on admission (March 19, 1915).

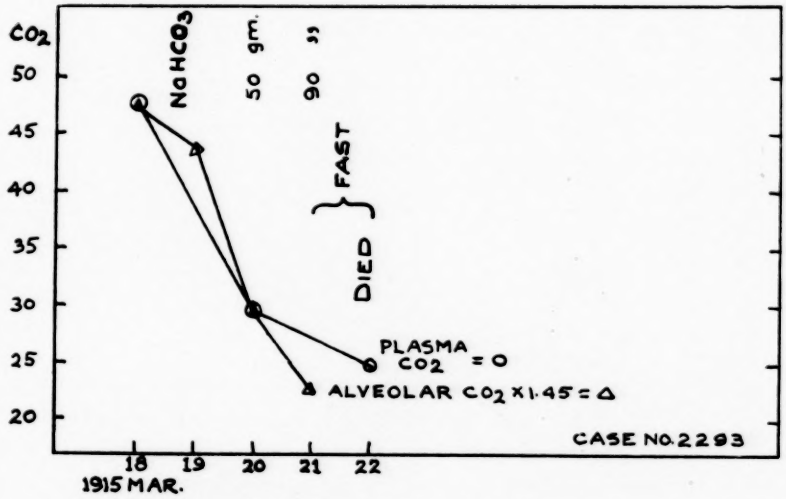
The curves are shown to illustrate the response of a case, on the border of coma to the fasting treatment with moderate doses of $NaHCO_3$. On this occasion the patient responded to such treatment. Chart 6 shows the diametrically opposite result obtained by fasting. These two charts taken together illustrate the necessity of having a patient under careful observation during the fasting period, and the impossibility of predicting the behavior even of a patient who has been fasted before.

(From March 19–May 4, 1915.) Group II.

8. Case 2293.

Date.	Total calories.	Protein.	Fat.	$NaHCO_3$.	Alcohol.	Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio $\frac{\text{plasma } CO_2}{\text{mm. alv. } CO_2}$	Plasma + 1 vol. 8.50 HCl.
1915		*gm.*	*gm.*	*gm.*	*cc.*	*mm.*	*mm.* × 1.45	*cc.*		*pH*
Mar.										
18	1,285	41.0	71.5		65	32.4	47.0	47.3	1.46	
19	1,815	62.5	115.0		70	30.0	43.5			
20*	1,635	53.0	123.0	50	25	20.5	29.7	25.9	1.26	
21*†	Fast day.			90	35	15.8	22.9			
22*†	" "							24.9 (Death.)		

* Drowsiness; hyperpnea; nausea.
† Moribund.



8. Case 2293, male, age 29. Diabetic 2 years, of the severest type in intolerance of any food. Complicated at the end by pulmonary tuberculosis.

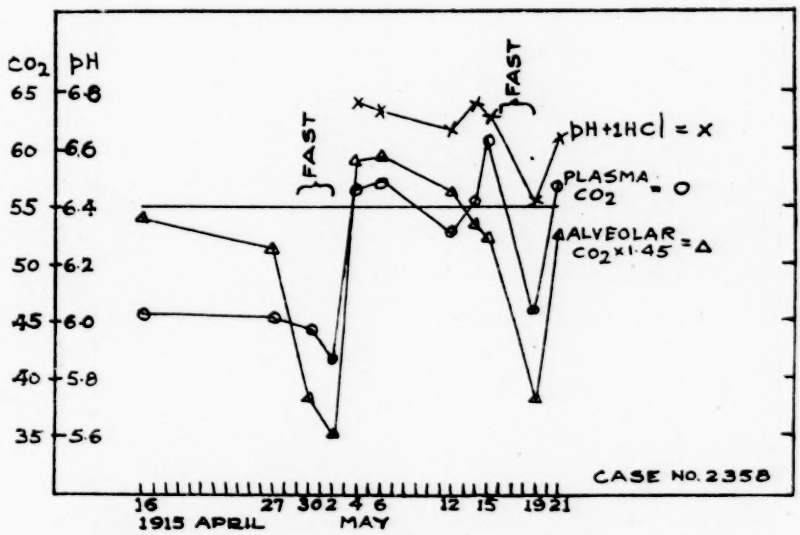
The curves show failure of response to large doses of $NaHCO_3$. Death occurred March 22, 1915.

(From March 18–22, 1915.) Group III–IV.

9. Case 2358.

Date.	Total calories.	Protein.	Fat.	Carbohydrate	Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio $\frac{\text{plasma } CO_2}{\text{mm. alv. } CO_2}$	Plasma + 1 vol. n/50 HCl.
1915		*gm.*	*gm.*	*gm.*	*mm.*	*mm. × 1.45*	*cc.*		*pH*
Apr. 16	1,915	62.5	178.5	0	37.2	54.0	45.8	1.23	
17	"	"	"						
18	Fast day.								
19	1,325	49.0	118.5	5					
20	"	"	"	"					
21	"	"	"	"					
22	"	"	"	"					
23	"	"	"	"					
24	"	"	"	"					
25	Fast day.								
26	1,350	50.0	119.0	10					
27	"	"	"	"	35.4	51.5	45.3	1.28	
28	325*	21.0	26.0						
29	Fast day.								
30	"	"			26.5	38.4	44.1	1.61	
May 1	"	"							
2	1,020	29.0	92.5	10	24.1	35.0	41.6	1.73	
3	1,350	50.0	119.0	"					
4	"	"	"	"	40.8	59.2	56.8	1.39	6.76
5	"	"	"	"					
6	1,375	52.0	"	15	40.7	59.0	57.0	1.40	6.73
7	1,495	52.5	131.5	"					
8	"	"	"	"					
9	Fast day.								
10	1,535	54.0	132.0	20					
11	"	"	"	"					
12	"	"	"	"	38.8	56.3	52.7	1.36	6.67
13	"	"	"	"					
14	1,595	57.0	133.0	30	36.8	53.4	55.2	1.50	6.76
15	250*	13.5	21.0		36.1	52.4	60.8	1.68	6.72
16	Fast day.								
17	"	"							
18	"	"							
19	715*	25.0	50.0	10	26.2	38.0	45.9	1.69	6.41
20	1,035	60.5	80.5	"					
21	"	"	"	"	36.0	52.2	56.9	1.58	6.64

*Partial fast.



9. Case 2358, age 11. Diabetic 1 year, of severe type in intolerance of any food.

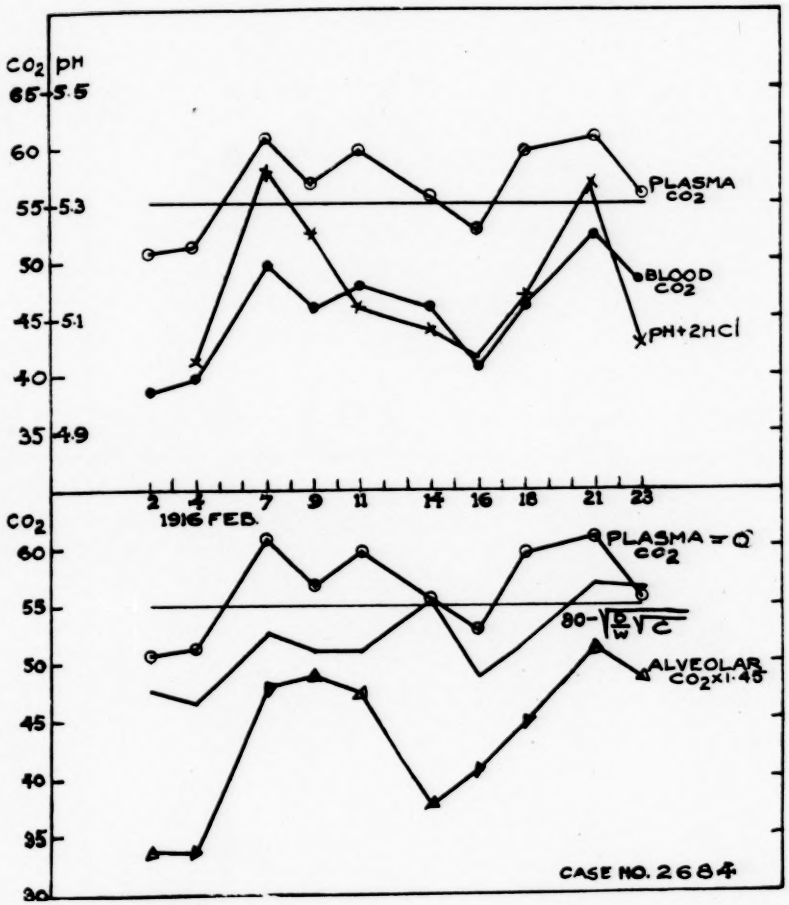
The curves show a sudden development of acidosis on two separate fasting periods of 3 days each with a return to normal on the resumption of diet.

(From April 16–May 21, 1915.) Group III.

10. Case 2684.

Date.	Diet.					Body weight.	Blood.						Urine.					
	Total calories.	Protein.	Fat.	Carbohydrate.	Alcohol.		Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio plasma CO_2 / mm. alv. CO_2	CO_2 bound by 100 cc. whole blood at 37°.	Plasma + 2 vol. N/50 HCl.	Vol. per 24 hrs.	Urea per liter.	NH_3 per liter.	Acid per liter.	$\sqrt{\frac{D}{W}}\sqrt{C}$	$80-\sqrt{\frac{D}{W}}\sqrt{C}$
1916		*gm.*	*gm.*	*gm.*	*cc.*	*kg.*	*mm.*	*mm.×1.45*	*cc.*		*cc.*	*pH*	*cc.*	*cc. N/10*	*cc. N/10*	*cc. N/10*		
Feb.																		
2	855	35	27.5	0	65	34.8			50.8		38.1		2,220	918	507	180	32.4	47.6
3	860	40	33.5	"	55													
4	855	45	38.5	"	45	35.3	23.1	33.5	51.1	2.21	39.7	5.01	1,790	1,425	662	152	33.5	46.5
5	850	50	43.0	"	35													
6	525	Fast day.			75													
7	850	40	40.0	"	45	35.5	33.0	47.9	60.6	1.84	49.3	5.36	1,665	1,580	532	116	27.2	52.8
8	"	"	"	"	"													
9	695	35	27.5	"	"	37.0	33.5	48.6	56.7	1.69	45.5	5.25	1,780	1,570	539	136	29.0	51.0
10	450*	12.5	9.5	"	"													
11	315	Fast day.			"	37.0	32.7	47.4	59.7	1.83	47.8	5.12	2,070	1,535	534	72	29.0	51.0
12	470*	14.5	10.5	"	"													
13	555	22.5	16.0	"	"													
14	575	27.5	"	"	"	37.0	26.1	37.8	55.2	2.11	45.6	5.08	2,280	964	365	90	24.5	55.5
15	605	32.5	16.5	"	"													
16	625	37.5	"	"	"	37.3	28.1	40.8	52.5	1.83	40.4	5.03	1,855	1,465	612	108	31.1	48.9
17	650	42.5	17.5	"	"													
18	"	"	"	"	"	38.0	30.9	44.8	59.3	1.92	46.0	5.13	2,345	1,225	445	70	28.3	51.7
19	"	"	"	"	"													
20	315	Fast day.			"													
21	650	42.5	17.5	"	"	37.2	35.3	51.2	60.9	1.72	52.1	5.34	1,545	1,408	470	84	23.0	57.0
22	680	35.0	24.0	"	"													
23	710	40.0	"	"	"	38.0	33.6	48.7	55.6	1.65	47.3	5.05	1,965	1,415	399	96	24.0	56.0

*Partial fast.



10. Case 2684, female, age 43.

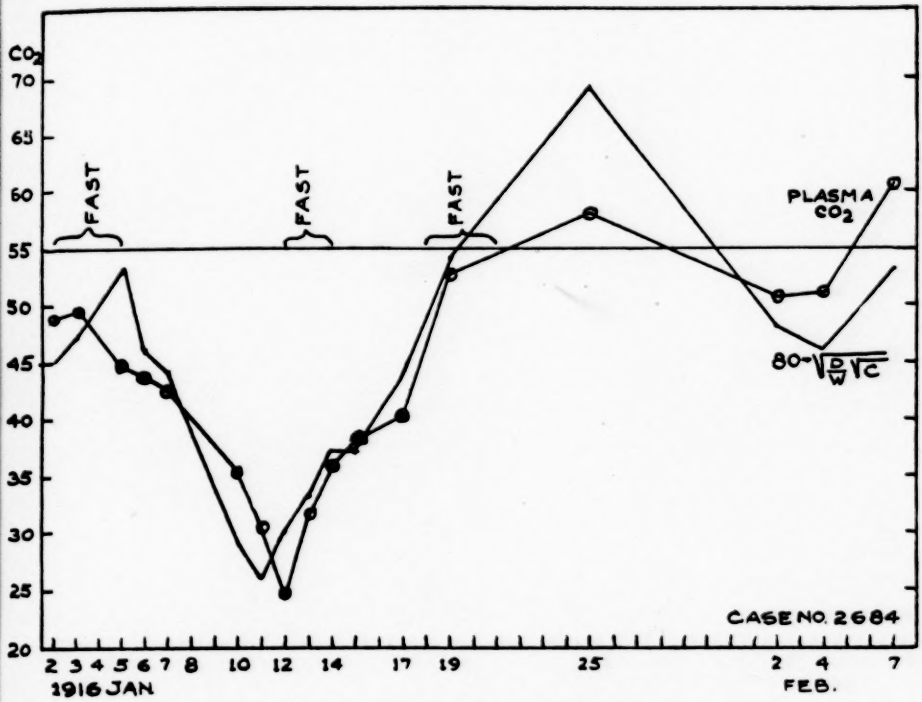
The curves show a continuous low grade acidosis, possibly caused by a very low protein-fat (carbohydrate-free) diet, necessitated by a persistent glycosuria. The low level of these curves was subsequently noted over a period of 4 months' observation. The acid excretion corresponds to the plasma bicarbonate, but the alveolar CO_2 was consistently very much too low.

(From February 2–23, 1916.) Group III.

11. Case 2684.

Date.	Diet.					Body weight.	Blood.	Urine.					
	Total calories.	Protein.	Fat.	Carbohydrate.	Alcohol.		CO_2 bound by 100 cc. plasma at 20°.	Vol. per 24 hrs.	Urea per liter.	NH_3 per liter.	Acid per liter.	$\frac{D}{W}\sqrt{C}$	$89-\frac{D}{W}\sqrt{C}$
1916		*gm.*	*gm.*	*gm.*	*cc.*	*kg.*	*cc.*	*cc.*	*cc. N/10*	*cc. N/10*	*cc. N/10*		
Jan.													
2	Fast day.					36.0	47.8	2,150	2,120	530	230	35.0	45.0
3	" "					35.3	48.2	1,615	1,475	579	250	33.0	47.0
4	" "												
5	160	15	10.5	0		35.0	44.5	2,170	1,352	350	165	27.0	53.0
6	320	30	21.0	"		34.2	43.8	1,830	2,010	602	195	34.0	46.0
7	1,075	44.5	52.0	"	60	34.7	42.5	2,285	2,283	596	165	36.0	44.0
8	1,525	60.0	92.5	"	"								
9	"	"	"	"	"								
10	2,145	74.5	153.0	"	"	34.1	35.2	1,975	2,375	942	325	51.0	29.0
11	1,830	"	"	"	15	34.3	30.4	2,120	2,695	964	345	54.0	26.0
12*	Fast day.					34.7	24.6	3,355	998	678	200	50.0	30.0
13	" "					33.6	31.6	2,720	785	701	185	47.0	33.0
14	160	15	10.5	0		33.3	35.9	2,300	1,010	709	148	43.0	37.0
15	240	22.5	16.0	"		33.3	38.1	2,210	1,358	734	180	43.0	37.0
16	Fast day.												
17	500*	7.5	5.5	0	60	33.2	40.0	2,520	848	574	105	36.0	44.0
18	420	Fast day.											
19	"	" "			"	33.2	52.8	1,440	1,550	540	85	26.0	54.0
20	"	" "			"								
21	420	Green veg. only containing			10	"							
22	525	" " "	"		20	75							
23	"	" " "	"		"	"							69.0
24	"	" " "	"		25	"							
25	"	" " "	"		30	"	33.6	58.0	2,155	850	98	60	11.0	

* Drowsiness; hyperpnea; nausea.



11. Case 2684, female, age 43 (the same case as No. 10). Diabetic of extreme severity with marked emaciation. A mild acidosis was present on admission (January 1, 1916).

The curves show a dangerous response to a moderate protein-fat (carbohydrate-free) diet, which was accompanied by clinical manifestations of severe acidosis. At the end of 9 days' fasting the patient may be considered past danger from an acidosis point of view, as is indicated by the rise of the curves. The curves show consistent parallelism between acid excretion and plasma bicarbonate.

(From January 2–25, 1916.) Group II.

12. Case 2128.

Date.	Diet.					Body weight.	Blood.		Urine.					
	Total calories.	Protein.	Fat.	Carbohydrate.	$NaHCO_3$.		CO_2 bound by 100 cc. plasma at 20°.	CO_2 bound by 100 cc. whole blood at 37°.	Vol. per 24 hrs.	Urea per liter.	NH_3 per liter.	Acid per liter.	$\sqrt{\frac{D}{W}}\sqrt{C}$	$80 - \sqrt{\frac{D}{W}}\sqrt{C}$
1916		*gm.*	*gm*	*gm.*	*gm.*	*kg.*	*cc.*	*cc.*	*cc.*	*cc. N/10*	*cc. N/10*	*cc. N/10*		
Mar. 20	625	40	47.5	7										
21*	Fast day.				30	37.0	33.3	29.9	7,740	390	312	102	42.0	38.0
22	" "					36.9	60.1	48.4	4,400	456	315	48	28.7	55.3
23	" "					37.0	45.9	38.8	3,995	442	388	68	33.4	46.6
24	120†	20	3.5			36.6	48.2	38.4	3,635	524	385	40	29.4	50.6
25	800	40	68.5			36.8	51.5	45.0	3,210	840	431	50	30.3	49.7
26	655	50	48.5											
27	600	70	33.5			37.5	50.2	42.4	3,590	1,225	412	76	31.9	48.1
28	Green veg. only containing			10		38.0	52.6	43.5	4,980	500	232	38	24.5	55.5
29	" " " "			20		37.7	58.3	46.7	4,740	454	200	36	21.2	58.8
30	" " " "			30										
31	" " " "			40		36.4	63.6	52.0	5,145	400	94	24	13.3	66.7

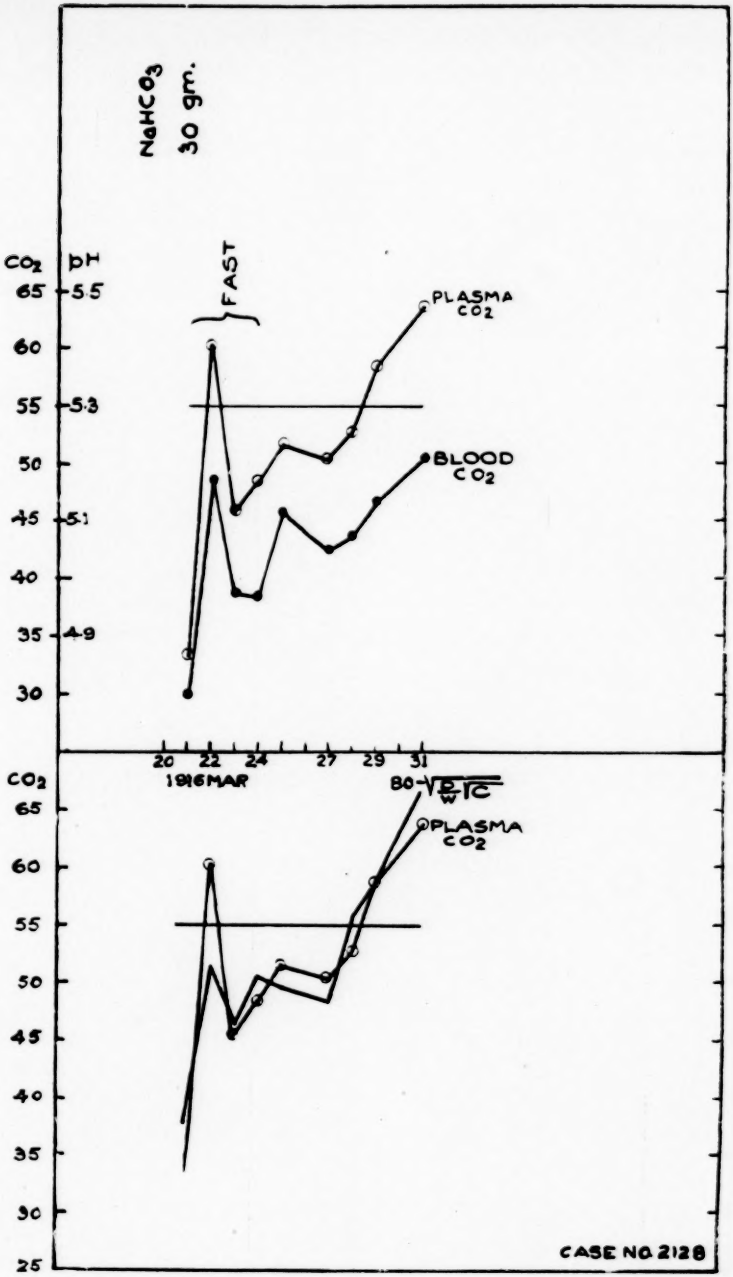
* Drowsiness.

† Partial fast.

12. Case 2128, female, age 19. Diabetic 4 years, of extreme severity in the inability to metabolize carbohydrate or fat, but without marked emaciation or loss of strength. Over 2 years of observation has shown a constant low grade acidosis.

The curves show the dangerous effect of the unrestricted diet indulged in prior to her readmission, with the striking result gained by fasting and a carbohydrate tolerance test. The parallelism between acid excretion and plasma bicarbonate was also striking.

(From March 20–31, 1916.)

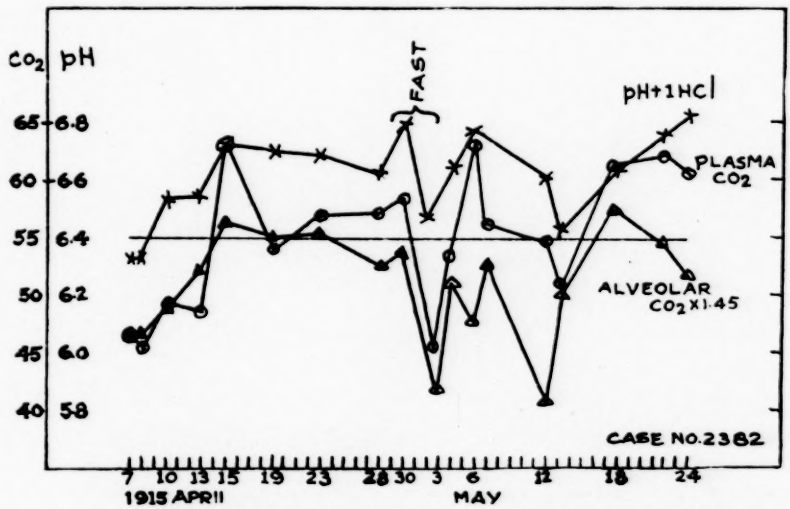

NaHCO3
30 gm.
CO2
pH
65
60
55
50
45
40
35
30
5.5
5.3
5.1
4.9
FAST
PLASMA CO2
BLOOD CO2
20
22
24
27
29
31
1916 MAR
80-√D/W TC
PLASMA CO2
25
CASE NO 2128

Date.	Total calories.	Protein.	Fat.	Carbohydrate.	Alcohol.	Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio $\frac{\text{plasma } CO_2}{\text{mm. alv. } CO_2}$	Plasma + 1 vol. s/50 HCl.
1915		*gm.*	*gm.*	*gm.*	*cc.*	*mm.*	*mm.×1.45*	*cc.*		*pH*
Apr.										
7	Green veg. only containing			15.0	15	32.0	46.4	46.3	1.45	6.33
8	" " " "			20.0		32.2	46.7	45.3	1.41	6.32
9	" " " "			30.0						
10	" " " "			49.0		33.5	48.6	49.0	1.46	6.55
11*	" " " "			19.0						
12	2,440	85.5	224.5							
13	2,440	92.5	222.0			35.9	52.1	48.4	1.35	6.55
14	2,500	97.0	226.5							
15	2,440	88.0	224.0			38.8	56.2	63.1	1.63	6.73
16	2,440	92.5	121.5							
17	2,440	85.5	224.5							
18	Fast day.									
19	2,440	92.5	221.5			38.0	55.1	54.0	1.42	6.70
20	2,440	92.5	221.5							
21	2,440	85.5	224.5							
22	2,420	93.0	219.5							
23	2,440	85.5	224.5			38.3	55.6	56.0	1.46	6.68
24	2,440	92.5	221.5							
25	Fast day.									
26	2,210	92.5	196.5							
27	2,210	92.5	196.5							
28	2,185	93.0	194.5			36.4	52.9	56.0	1.53	6.62
29	Fast day.									
30	" "					37.0	53.7	57.3	1.55	6.80
May										
1	" "									
2	" "									
3	Green veg. only containing			20.0		28.7	41.6	45.1	1.57	6.46
4	" " " "			40.0		35.2	51.0	53.2	1.52	6.64
5	" " " "			50.0						
6	" " " "			60.0		32.7	47.4	63.0	1.93	6.75
7	" " " "			70.0		36.4	52.8	56.0	1.54	
8	" " " "			80.0						
9	" " " "			90.0						
10	" " " "			100.0						
11*	" " " "			24.5						

13. Case 2382—Concluded.

Date.	Total calories.	Protein.	Fat.	Carbohydrate	Alcohol.	Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio plasma CO_2 mm. alv. CO_2	Plasma + 1 vol. — N/50 HCl.
1915		*gm.*	*gm.*	*gm.*	*cc.*	*mm.*	*mm.* × *1.45*	*cc.*		*pH*
May										
12	555	24.0	44.5	10.0		27.8	40.3	54.5	1.96	6.61
13	930	43.0	72.0	20.0		34.4	49.9	50.9	1.48	6.42
14	1,230	47.0	105.5	15.0						
15	1,370	62.0	113.5	15.0						
16	555*	21.5	49.0	3.0						
17	935*	46.5	80.0	12.5						
18	1,530	78.0	123.0	15.0		39.3	57.3	61.3	1.56	6.64
19	1,520	60.5	130.5	15.0						
20	1,450	70.0	123.5	3.5						
21	1,430	71.0	122.5							
22	1,425	60.5	127.0			37.7	54.7	62.3	1.65	6.76
23	Fast day.									
24	1,425	53.0	130.0			35.6	51.6	60.6	1.70	6.83

*Partial fast.



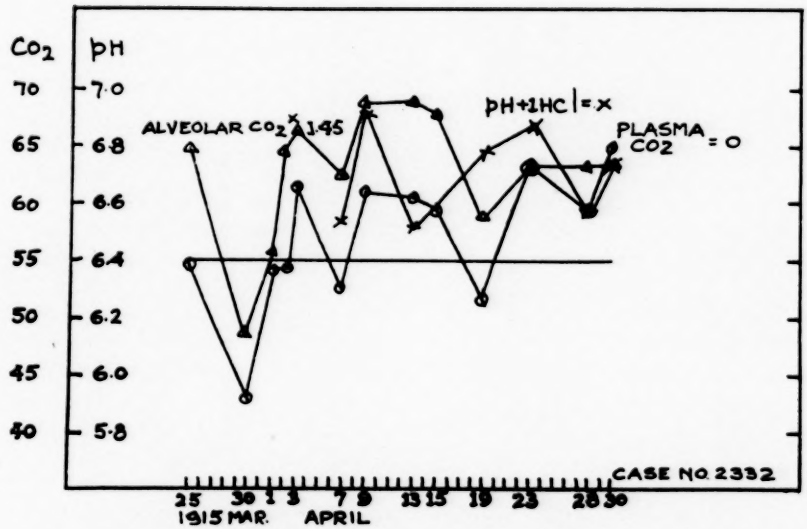
13. Case 2382, male, age 40. Diabetic 8 years, severe in intolerance of food, with a tendency to a low grade chronic acidosis.

The curves are peculiar in that they are high during a period of relatively high fat feeding, and show an unstable and low level during a carbohydrate tolerance test, due perhaps to the 4 days' fast which preceded this test.

(From April 7–May 24, 1915.) **Group III.**

14. Case 2332.

Date.	Total calories.	Protein.	Fat.	Carbohydrate.	Alcohol.	Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio $\frac{\text{plasma } CO_2}{\text{mm. alv. } CO_2}$	Plasma + 1 vol. $\frac{N}{50}$ HCl.
1915		*gm.*	*gm.*	*gm.*	*cc.*	*mm.*	*mm.×1.45*	*cc.*		*pH*
Mar.										
25	1,720	91.5	58	30	97.5	44.7	64.8	54.6	1.22	
26	1,725	85.5	61	"	"					
27	"	91.5	58.5	"						
28	682	Fast day.			"					
29	2,180	111.0	99.0	30	"					
30	2,215	111.5	102.5	"	"	33.3	48.3	42.7	1.28	
31	2,250	112.5	105.5	"	"					
Apr.										
1	2,215	112.0	102.5	"	"	38.5	55.8	54.4	1.41	
2	2,385	114.0	115.0	40	"	44.6	64.7	54.4	1.22	
3	"	"	"	"	"	45.9	66.6	61.5	1.34	
4	700	Fast day.			100.0					
5	2,370	116.0	111.0	40	"					
6	2,410	114.0	116.0	"	"					
7	2,890	115.0	167.0	"	"	42.9	62.2	52.4	1.22	6.46
8	2,660	"	"	"	67.5					
9	1,920	88.0	150.0	"		47.6	69.0	61.0	1.28	6.92
10	1,975	86.0	156.5	"						
11	157	Fast day.			22.5					
12	1,975	87.0	156.5	40						
13	2,025	90.5	156.0	50		47.6	69.0	60.6	1.27	6.50
14	2,060	90.0	160.0	"						
15	2,085	91.5	173.5	"		46.8	67.7	59.5	1.27	6.59
16	2,145	90.5	169.0	"						
17	2,095	89.0	164.0	"						
18	Fast day.									
19	2,035	91.5	156.5	50		40.1	58.2	51.4	1.28	6.78
20	1,975	89.0	155.5	40						
21	2,010	79.0	174.5	"						
22	1,955	89.0	153.5	"						
23	1,945	87.5	"	"		43.5	63.1	63.1	1.45	6.84
24	1,975	90.0	155.5	"						
25	Fast day.									
26	1,970	90.0	161.0	25						
27	2,000	"	165.0	"						
28	2,165	93.5	180.5	"		43.6	63.2	59.0	1.35	6.58
29	2,110	93.0	175.0	"						
30	2,140	92.5	178.5	"		43.7	63.4	64.9	1.49	6.74

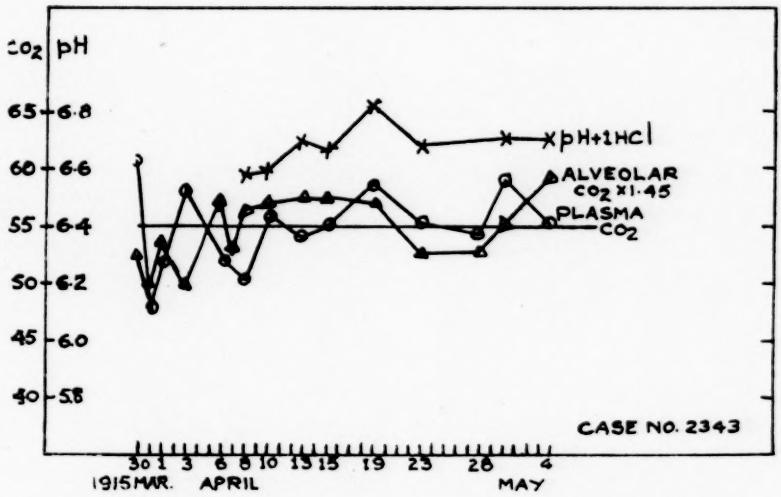


14. Case 2332, age 42. Diabetic 8 years, of mild type, complicated by furunculosis and carbunculosis.

During the period over which the chart is drawn, the patient suffered a rather intense gastrointestinal upset of unknown origin. This is reflected in the instability of the curves. Following the recovery from this attack, the curves continue on a high level up to the time of discharge from the hospital.

(From March 25–April 30, 1915.) Group I.

Date.	Total calories.	Protein.	Fat.	Carbohydrate.	Alcohol.	Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio $\frac{\text{plasma } CO_2}{\text{mm. alv. } CO_2}$	Plasma + 1 vol. n/50 HCl.
1915		*gm.*	*gm.*	*gm.*	*cc.*	*mm.*	*mm.* × *1.45*	*cc.*		*pH*
Mar.										
30	1,940	98.5	83.5	15.0	100	36.2	52.2	60.5	1.67	
31	1,940	80.5	91.0	"	100	33.9	49.2	47.8	1.41	
Apr.										
1	1,965	100.0	84.0	20.0	100	37.1	53.8	51.6	1.39	
2	1,965	82.0	91.0	"	100					
3	1,970	89.5	88.0	"	100	34.4	49.9	58.2	1.69	
4	1,960	97.0	84.5	"	100					
5	2,235	108.5	109.0	19.0	100					
6	2,245	120.0	104.5	20.0	100	39.3	57.0	52.0	1.32	
7	2,240	102.0	112.0	"	100	36.4	52.8	51.4	1.41	
8	2,065	102.0	112.0	"	75	38.8	56.2	49.3	1.27	6.57
9	1,490	97.5	104.0	30.0						
10	1,510	98.5	105.5	"		39.3	57.0	56.0	1.42	6.58
11	210	Fast day.			30					
12	1,725	97.5	129.5	30.0						
13	1,955	90.0	157.5	"		39.8	57.7	54.0	1.36	6.69
14	1,970	92.0	158.5	"						
15	2,205	92.0	183.5	"		39.5	57.3	55.0	1.39	6.66
16	2,090	97.0	179.5	"						
17	2,085	89.5	182.5	"						
18	Fast day.									
19	2,215	90.0	180.5	40.0		39.3	57.0	58.5	1.49	6.82
20	2,745	104.0	231.5	"						
21	2 730	95.0	234.0	"						
22	2,715	102.5	229.0	"						
23	2,710	101.5	229.0	"		36.9	52.5	55.2	1.50	6.68
24	2,715	102.5	230.0	"						
25	Fast day.									
26	2,665	97.0	231.0	30.0						
27	2,655	97.0	230.0	"						
28	2,775	90.0	245.5	"		36.4	52.8	54.4	1.49	
29	2,755	96.5	240.5	"						
30	2,760	92.0	243.0	"		38.0	55.1	59.0	1.55	6.70
31	2,735	94.0	239.0	"						
May 1	Fast day.									
2	2,760	93.5	242.0	30.0						
3	2,780	97.0	243.0	"						
4	2,930	101.0	257.5	"		40.8	59.2	55.4	1.36	6.70
5	3,090	104.0	273.5	"						
6	3,090	104.0	273.5	"						
7	2,580	100.5	220.0	"						



15. Case 2343, male, age 44. Diabetic 9 years, mild in type, without evidence of acidosis before the institution of active treatment.

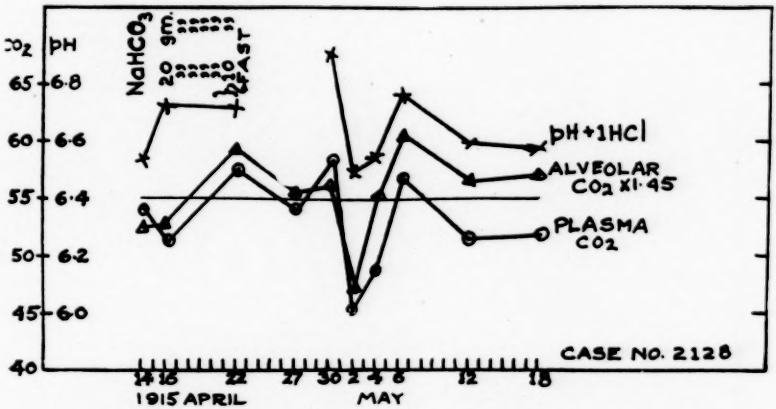
The curves show an unstable and rather low level, following a 2 months' period of very low diet, and a gradual rising and uniformity of the curves when the patient was placed on an adequate diet.

(From March 30–May 4, 1915.) Group I.

16. Case 2128.

Date.	Total calories.	Protein.	Fat.	Carbohydrate.	$NaHCO_3$.	Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio plasma CO_2 mm. alv. CO_2	Plasma + 1 vol. n/50 HCl.
1915		*gm.*	*gm.*	*gm.*	*gm.*	*mm.*	*mm. × 1.45*	*cc.*		*pH*
Apr.										
15	2,040	142	145	26.5	20	35.9	52.1	54.0	1.50	6.53
16	2,830	202.5	204	26.0	"	36.4	52.8	51.4	1.41	6.72
17	2,780	155.0	223	18.0	"					
18	1,845	129.0	133	19.0	"					
19	1,370	47.0	104.5	50.5	"					
20	Fast day.				"					
21	" "				10					
22	2,005	8.0	201.5	24.0		40.8	59.2	57.5	1.41	6.72
23	2,225	32.5	186.0	89.5						
24	2,430	40.5	202.0	95.0						
25	1,000	39.0	58.0	74.0						
26	Green veg. only containing			75.0						
27	"					38.3	55.5	54.0	1.41	
28	1,855	2.6	155.5	75.0						
29	"	"	"	"						
30	930	0	100.0	0		38.7	56.1	58.8	1.52	6.92
May										
1	1,440	29.5	141.5	1.0						
2	1,450	30.5	142.0	"		31.3	45.4	45.1	1.44	6.49
3	1,075	"	102.0	"						
4	1,040	26.0	100.0	"		38.0	55.1	48.6	1.28	6.55
5	815	24.0	80.0	"						
6	1,095	37.0	78.0	54.0		42.0	60.9	57.0	1.36	6.78
7	Fast day.									
8	1,095	32.5	67.0	83.0						
9	1,075	33.0	66.5	67.5						
10	1,060	29.0	66.0	81.0						
11*	175	Fast day.								
12	870	26.5	44.0	86.5		38.8	56.3	51.8	1.35	6.60
13	930	32.5	40.0	104.0						
14	875	29.5	40.0	93.0						
15	1,180	45.0	45.5	140.0						
16	465	0	50.0	0						
17	1,130	43.5	46.5	124.5						
18	950	42.0	29.0	123.5		39.3	57.0	51.9	1.32	6.57

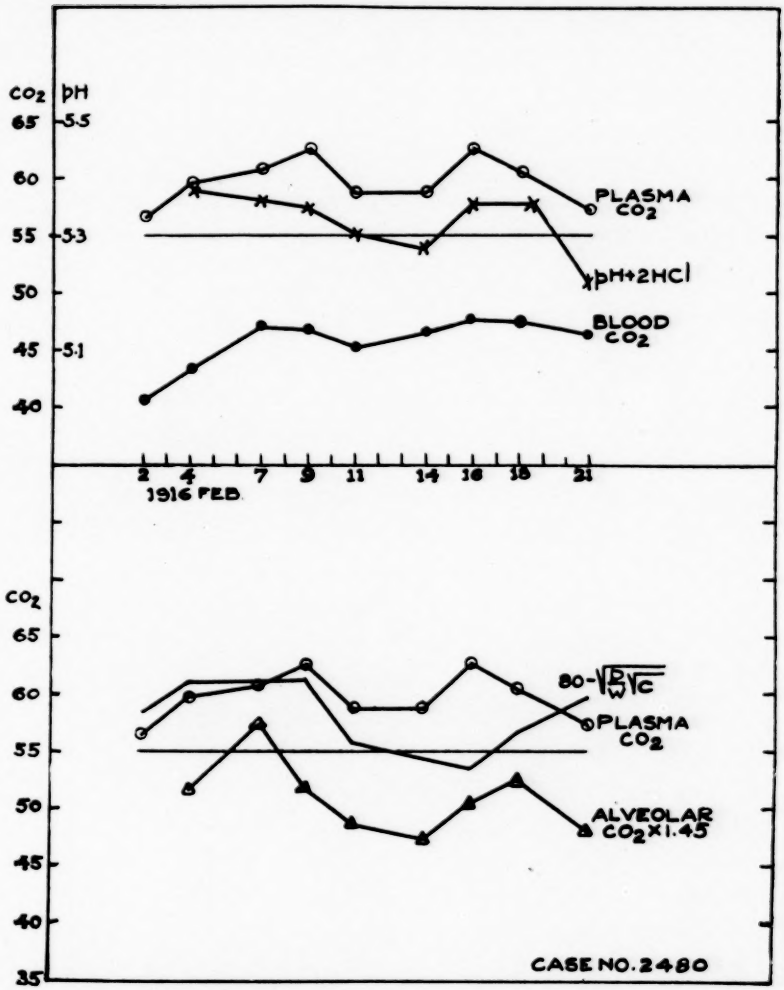
*Alcohol, 25 cc.



16. Case 2128, female, age 18. Diabetic 3 years, of severe type in inability to metabolize carbohydrate or fat, though maintaining a fair state of nutrition and strength.
(From April 15–May 18, 1915.) Group III–IV.

17. Case 2480.

Date.	Diet.				Body weight.	Blood.						Urine.					
	Total calories.	Protein	Fat.	Carbohydrate.		Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio $\frac{\text{plasma } CO_2}{\text{alv. } CO_2}$	CO_2 bound by 100 cc. whole blood at 37°.	Plasma $+\frac{2}{\text{vol.}}$ N/50 HCl	Vol. per 24 hrs.	Urea per liter.	NH_3 per liter.	Acid per liter.	$\frac{D}{W\sqrt{C}}$	$80-\frac{D}{W\sqrt{C}}$
1916		*gm.*	*gm.*	*gm.*	*kg*	*mm.*	*mm. × 1.45*	*cc.*		*cc.*	*pH*	*cc.*	*cc. N/10*	*cc. N/10*	*cc. N/10*		
Feb.																	
2	1,400	75	120	0	40.8			56.3		40.5		1,945	2,975	300	125	21.8	58.2
3	"	"	115	2.5													
4	"	"	"	"	40.5	35.5	51.3	59.6	1.68	43.1	5.38	2,065	3,435	251	120	19.0	61.0
5	"	"	"	"													
6	Fast day.																
7	1,300	65	110	0	39.1	39.4	57.2	60.6	1.54	47.0	5.36						
8	"	"	"	"													
9	"	"	"	"	40.8	39.2	51.8	62.4	1.59	46.8	5.35	2,195	3,000	275	120	18.8	61.2
10	"	"	"	"													
11	"	"	"	"	41.0	33.5	48.6	58.8	1.76	45.0	5.31	1,250	4,620	540	180	24.2	55.8
12	"	"	"	"													
13	Fast day.																
14	1,300	65	110	0	40.8	32.7	47.4	58.8	1.80	46.6	5.28	920	4,890	540	184	20.9	59.1
15	"	"	"	"													
16	"	"	"	"	41.4	34.6	50.2	62.7	1.81	47.7	5.36	1,625	3,685	460	132	26.7	53.3
17	"	"	"	"													
18	"	"	"	"	41.8	36.0	52.2	60.3	1.68	47.4	5.36	1,095	5,010	586	196	23.5	56.5
19	"	"	"	"													
20	Fast day.																
21	1,100	65	90	0	41.4	33.4	48.0	57.1	1.71	46.3	5.22	1,400	3,375	433	140	20.4	59.6



17. Case 2480, female, age 28. Diabetic 5 years, formerly of a mild type, but latterly developing into an obstinately progressive form, with emaciation and inability to tolerate calories enough to maintain existence in an aglycosuric state. A low grade acidosis has been present for 15 months of observation.

During the period shown the patient was temporarily free from acidosis as the result of unusual response to treatment. The acid excretion corresponds to the plasma bicarbonate, but the alveolar CO_2 is consistently too low.

(From February 2–21, 1916.) Group III.

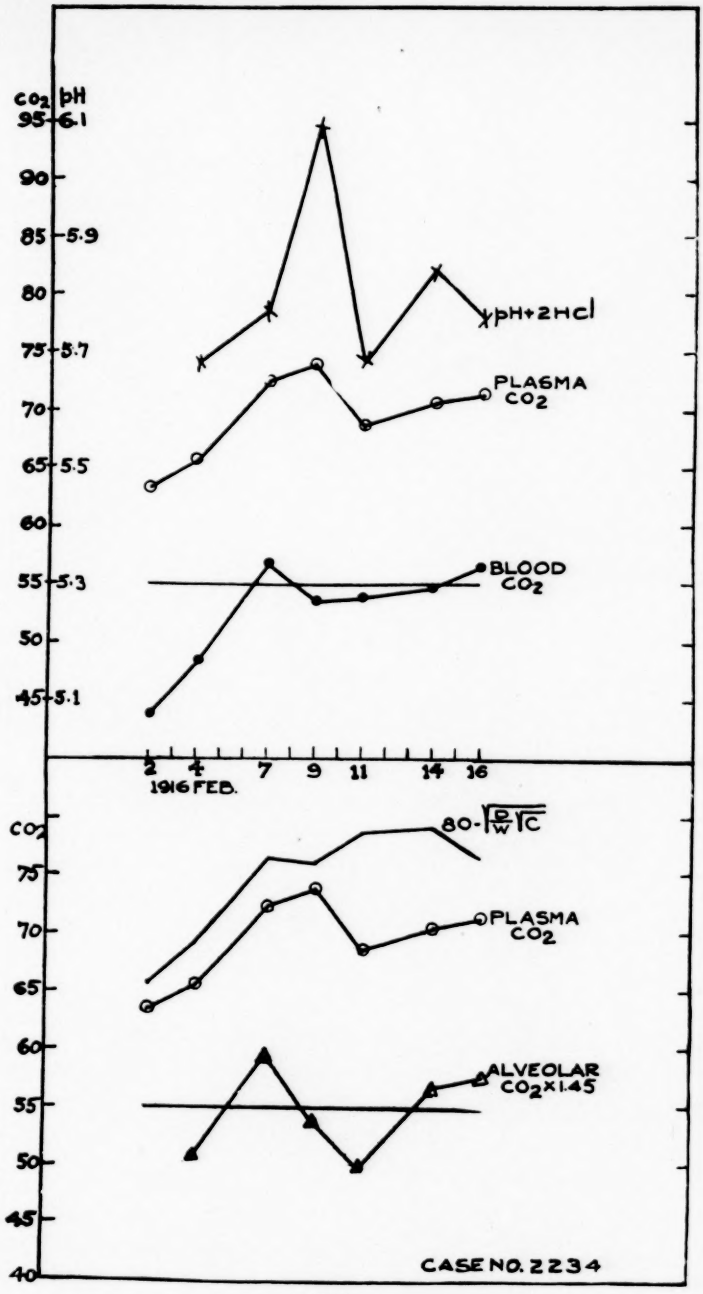
18. Case 2234.

Date.	Diet.				Body weight.	Blood.						Urine.					
	Total calories.	Protein.	Fat.	Carbohydrate.		Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio plasma CO_2 mm. alv. CO_2	CO_2 bound by 100 cc. whole blood at 37°.	Plasma + 2 vol. N/50 HCl	Vol. per 24 hrs.	Urea per liter.	NH_3 per liter.	Acid per liter.	$\frac{D}{W}\sqrt{C}$	$90-\frac{D}{W}\sqrt{C}$
1916		*gm.*	*gm.*	*gm.*	*kg.*	*mm.*	*mm. × 1.45*	*cc.*		*cc.*	*pH*	*cc.*	*cc. N/10*	*cc. N/10*	*cc. N/10*		
Feb.																	
2	Green veg. only containing			20	48.0			63.1		43.7		740	3,380	270	300	14.2	65.8
3	"	"	"	"	30												
4	"	"	"	"	40	48.2	34.8	50.4	65.5	1.88	48.2	5.68	1,050	3,080	128	184	10.8	69.2
5	"	"	"	"	50													
6	"	"	"	"	60													
7	"	"	"	"	70	48.0	40.9	59.3	72.3	1.77	56.3	5.77	2,275	1,575	22	34	3.4	76.6
8	"	"	"	"	80													
9	"	"	"	"	90	48.2	37.0	53.6	73.9	2.0	53.2	6.09	1,975	1,460	22	34	4.0	76.0
10	"	"	"	"	100													
11	"	"	"	"	110	48.6	34.2	49.6	68.8	2.01	53.9	5.68	1,890	1,525	23	26	1.8	78.2
12	"	"	"	"	120													
13	"	"	"	"	130													
14	"	"	"	"	140	48.6	39.2	56.8	70.4	2.80	54.8	5.84	2,145	1,460	18	22	1.0	79.0
15	"	"	"	"	150													
16	"	"	"	"	115	49.2	39.6	57.4	71.1	2.80	56.1	5.76	1,635	2,030	20	32	3.0	77.0

18. Case 2234, male, age 54. Diabetic 4 years, of the arteriosclerotic underweight type, albuminuric and cylindruric, but with a normal urea index.

The chart shows the curve of typical mild diabetes without acidosis, on the diet of a carbohydrate tolerance test. (From February 2–16, 1916.)

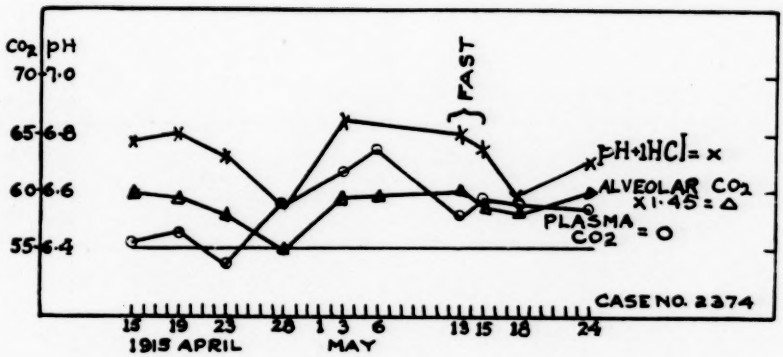
Group I


CO_2 pH
95 6.1
90
85 5.9
80
75 5.7
70
65 5.5
60
55 5.3
50
45 5.1
pH+2HCl
PLASMA CO_2
BLOOD CO_2
2 4 7 9 11 14 16
1916 FEB.
CO_2
80-$\sqrt{\frac{D}{W}C}$
75
70
65
60
55
50
45
40
PLASMA CO_2
ALVEOLAR CO_2 X 1.45
CASE NO. 2234

19. Case 2374.

Date.	Total calories.	Protein.	Fat.	Carbohydrate	Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio $\frac{\text{plasma } CO_2}{\text{mm. alv. } CO_2}$	Plasma + 1 vol. n/50 HCl.
1915		*gm.*	*gm.*	*gm.*	*mm.*	*mm. × 1.45*	*cc.*		*pH*
Apr. 15	Green veg. only containing			10	41.0	59.5	55.4	1.35	6.77
16	" " "		"	"					
17	" " "		"	"					
18	Fast day.								
19	700	25	57.5	15	40.8	59.2	56.4	1.38	6.80
20	1,190	44.5	101.7	"					
21	"	"	"	"					
22	1,380	63.5	114.0	"					
23	"	"	"	"	39.8	57.8	33.4	1.34	6.72
24	1,325	65.0	107.5	"					
25	Fast day.								
26	1,550	65.5	131.5	15					
27	1,605	64.0	138.0	"					
28	1,530	66.0	130.0	"	37.7	54.7	59.0	1.57	6.53
29	"	"	"	"					
30	1,595	"	136.0	"					
May 1	1,540	73.5	126.5	"					
2	Fast day.								
3	1,625	75.0	133.0	20	40.8	59.2	61.8	1.51	6.85
4	1,665	"	137.0	"					
5	1,705	67.5	145.5	"					
6	2,090	76.0	182.0	"	41.0	59.5	63.6	1.55	
7	1,975	72.5	175.0	13					
8	1,820	68.5	165.5	0					
9	Fast day.								
10	1,910	66.5	169.5	15					
11	503*	20.5	44.0	2					
12	Fast day.								
13	" "				41.4	60.0	57.7	1.39	6.80
14	" "								
15	675*	32.5	54.0	10	40.4	58.6	59.0	1.46	6.75
16	1,040	45.0	98.0	"					
17	1,250	63.5	102.5	"					
18	"	"	"	"	40.0	58.0	58.3	1.46	6.57
19	"	"	"	"					
20	1,260	72.5	99.5	"					
21	1,575	75.0	132.0	"					
22	1,805	83.5	153.5	"					
23	Fast day.								
24	1,570	57.0	140.0	10	40.4	60.0	58.5	1.41	6.71

*Partial fast.



19. Case 2374, age 61. Diabetic 11 years, of a mild type, apparently arrested until the year before admission, when, following a physical shock, the patient showed signs of marked acidosis.

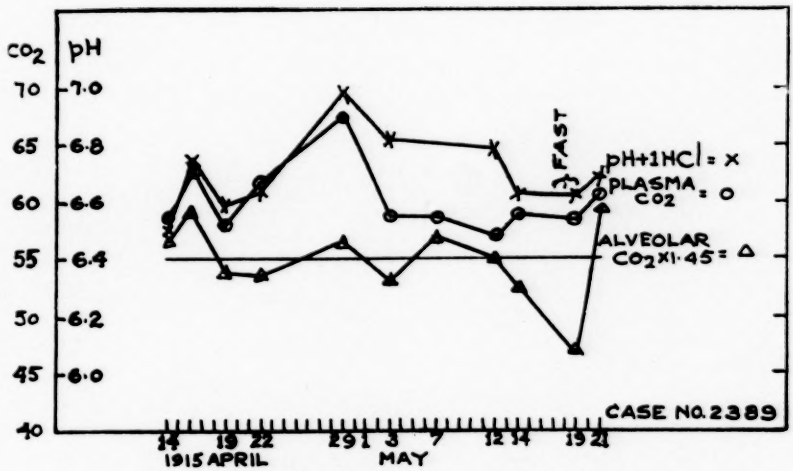
The curves illustrate the absence of acidosis in a mild diabetic responding satisfactorily to treatment.

(From April 15–May 24, 1915.) Group I.

20. Case 2389.

Date.	Total calories.	Protein.	Fat.	Carbohydrate.	Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio $\frac{\text{plasma } CO_2}{\text{mm. alv. } CO_2}$	Plasma + $\frac{1}{10}$ vol. $\frac{n}{50}$ HCl.
1915		*gm.*	*gm.*	*gm.*	*mm.*	*mm.* × *1.45*	*cc*		*pH*
Apr.									
14	1,805	46.5	165	20	38.8	56.2	58.5	1.51	6.50
15	1,860	50.0	"	30					
16	1,815	56.5	"	20	40.7	59.0	62.6	1.54	6.75
17	1,795	"	163.5	"					
18	Fast day.								
19	1,910	46.0	176.5	20	37.2	53.9	57.5	1.55	6.58
20	"	"	"	"					
21	"	"	"	"	37.1	53.8	61.6	1.66	6.65
23	"	"	"	"					
24	"	"	"	"					
25	Fast day.								
26	1,910	46.0	176.5	20					
27	"	"	"	"					
28	2,040	60.0	185.0	"					
29	2,025	67.0	180.5	"	39.0	56.6	67.2	1.72	6.99
30	"	"	"	"					
May									
1	1,910	66.0	167.5	"					
2	Fast day.								
3	1,995	70.0	170.0	30	36.5	53.0	58.7	1.61	6.81
4	"	"	"	"					
5	1,990	61.0	173.5	"					
6	2,020	60.0	177.5	"					
7	2,025	81.0	168.5	"	39.3	57.0	58.8	1.50	
8	2,020	70.5	173.0	"					
9	Fast day.								
10	2,020	70.5	173.0	30					
11	2,045	80.0	171.0	"					
12	2,040	62.5	178.5	"	37.9	55.0	57.0	1.50	6.79
13	1,920	65.5	169.0	20					
14	1,930	69.5	172.5	"	36.1	52.4	59.0	1.63	6.62
15	"	"	"	"					
16	400*	20.0	34.0						
17	Fast day.								
18	"	"							
19	1,450*	34.0	136.0	11	32.5	46.4	58.2	1.82	6.62
20	1,805	45.0	168.0	15					
21	"	"	"	"	41.0	59.5	60.6	1.48	6.69

*Partial fast.



20. Case 2389, male, age 35. Diabetic 4½ years, of moderate severity as shown by low tolerance for carbohydrate.

The curves show the absence of acidosis in a case which responds satisfactorily to treatment. The alveolar CO_2 following fasting on May 18–19, shows on the 19th a false drop, due not to fall in blood bicarbonate, but to the nervous effect of fasting.

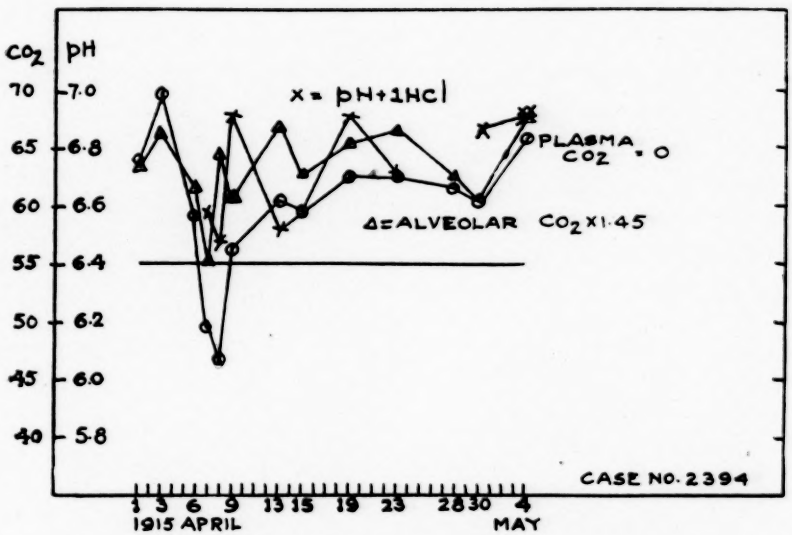
(From April 14–May 21, 1915.) Group I.

21. Case 2394.

Date.	Total calories.	Protein.	Fat.	Carbohydrate.	Alveolar CO_2.		CO_2 bound by 100 cc. plasma at 20°.	Ratio $\frac{\text{plasma } CO_2}{\text{mm. alv. } CO_2}$	Plasma + 1 vol. n/50 HCl.
1915		*gm.*	*gm.*	*gm.*	*mm.*	*mm.×1.45*	*cc.*		*pH*
Apr.									
1	1,175	34.5	50.0	150.0	43.6	63.2	63.2	1.45	
2	"	"	"	"					
3	1,300	40.5	45.0	175.0	45.9	66.7	69.8	1.52	
4	"	"	"	"					
5	Fast day.								
6	785	29.0	61.5	25.0	42.1	61.1	59.1	1.40	
7	1,290	50.0	106.0	"	37.7	54.7	49.4	1.31	6.59
8	1,425	70.0	111.5	"	44.6	64.7	46.3	1.04	6.47
9	1,780	69.0	150.0	"	41.7	60.5	56.4	1.35	6.92
10	"	"	"	"					
11	Fast day.								
12	2,110	76.0	171.5	50.0					
13	2,105	68.5	174.0	"	46.1	66.9	60.6	1.31	6.52
14	2,100	74.5	171.0	"					
15	2,545	71.0	220.0	"	43.2	62.6	59.5	1.38	6.60
16	2,585	76.5	221.0	"					
17	2,575	69.5	224.0	"					
18	Fast day.								
19	2,615	84.5	223.0	50	45.1	65.4	62.6	1.39	6.90
20	2,620	77.0	226.0	"					
21	2,640	80.0	228.5	"					
22	2,660	86.0	226.0	"					
23	2,710	95.5	217.5	"	45.7	66.3	62.1	1.36	6.71
24	2,715	85.0	232.5	"					
25	Fast day.								
26	2,590	84.0	219.5	50					
27	2,525	77.5	215.0	"					
28*	2,205	74.0	184.0	21	42.9	62.2	61.6	1.44	
29	Fast day.								
30	1,735**	60.5	161.0	22	41.5	60.2	60.3	1.45	6.86
May 1	2,385	80.5	210.5	25					
2	Fast day.								
3	2,380	80.0	210.0	25					
4	2,370	79.0	209.0	"	46.6	67.6	65.9	1.41	6.91

*Alcohol, 15 cc.

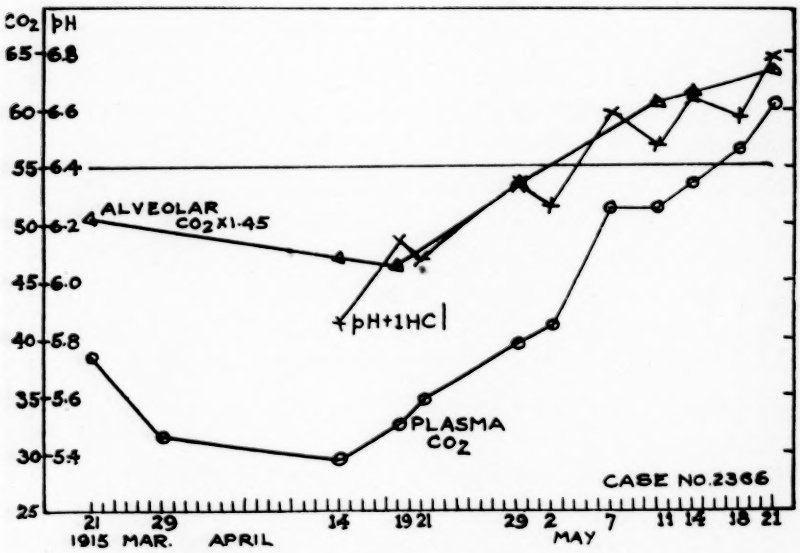
**Partial fast.



21. Case 2394, male, age 30. Diabetic 1¼ years, of mild type without any evidence of acidosis.

The chart shows a fall in the curves to below normal during the period of adjustment following a high carbohydrate diet. Following this period the curves remained normal throughout.

(From April 1–May 4, 1915.) Group I



22. Case 2366, male, age 26. Lobar pneumonia, empyema, acute nephritis. Acute nephritis was discovered February 12, 1915, during convalescence from a severe attack of lobar pneumonia followed by empyema. Typical course of severe acute nephritis with evidence of marked change in renal function followed recovery.

The chart shows the curves in a patient with the acidosis of acute nephritis, with return to normal as renal function improved. These curves are of especial interest because they show an effect of disease on respiration exactly opposite to that often encountered in severe diabetes. The alveolar CO_2 was consistently much too high to indicate the real extent to which the alkaline reserve was lowered.

(March 21–May 21, 1915.) Nephritic.

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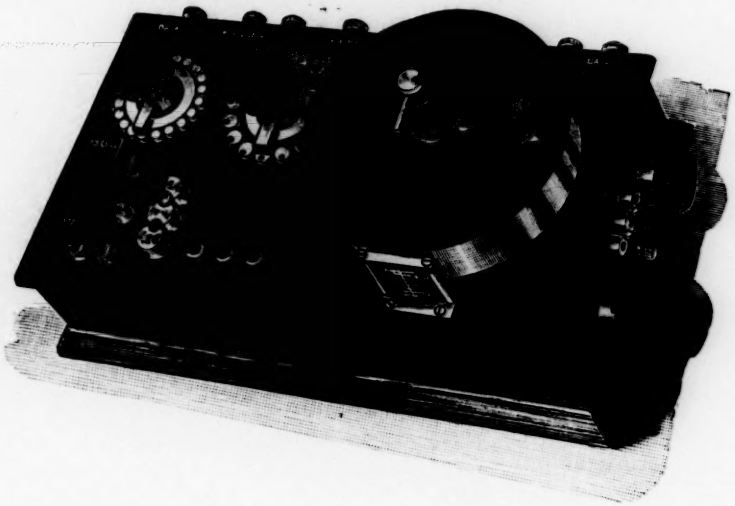

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